REMARKS

Status of the Claims

Prior to entry of the Amendment filed February 14, 2002, claims 1-43 are pending in this application. Claim 35 was cancelled in the Amendment filed February 14, 2002, and thus, after entry of that Amendment, claims 1-34 and 36-43 will be pending in the application. The amendments herein to claims 17 and 19 are supported by the specification, e.g., at page 6, line 25, to page 7, line 16, and page 23, lines 26-28. These amendments, which are not intended to limit the scope of the claims, add no new matter.

The rejection for lack of enablement

Claims 1-43 stand rejected under 35 U.S.C. §112, first paragraph, on the grounds that the specification allegedly does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims. Applicants respectfully traverse this rejection.

Applicants submit herewith a declaration of Daniel A. Vallera showing the *in vivo* efficacy of treatment of mice bearing a different tumor from that used in the experiment described in Example 6 of the instant application. Moreover, the targeting cells, the targeting domains, and one of the toxic domains used in the experiments described in the declaration are all different from those described in Example 6 of the instant specification. The targeting cells used in the experiments described in the declaration are CD4+ T cells while those used in the Example 6 were CD8+ T cells and the targeting domain used in the declaration experiments is IL-3 rather than IL-4 which was used in experiments described in Example 6 of the present application. In addition, the two molecules used as toxic domains (i.e., the DT390 fragment of diphtheria toxin and the mammalian proapoptotic protein BAX) in the experiments of the declaration are very different from each other, both structurally and in mechanism of action. Importantly, moreover, while the experiment described in Example 6 involved systemic (intravenous) administration of targeting cells to animals bearing a localized tumor, the experiments described in the declaration involve systemic (intraperitoneal) administration of targeting cells to mice with systemic (intraperitoneally administered) leukemia.

In that these data provide additional evidence for the ability of different targeting cells to direct functionally different immunotoxins to localized as well as systemically distrubuted pathogenic cells, they provide confirmatory support for the concept set forth throughout the instant specification (e.g., page 33; line 8, to page 38, line 14) that a variety of targeting cell types producing a variety of immunotoxins would be useful in a wide range of systemic pathogenic cell-mediated diseases such as autoimmune and infectious diseases as well as cancer.

Applicants submit, in addition, that one of skill in the art would conclude from the teaching of the instant specification that immunotoxic therapy in which a particular immunotoxin is targeted to a disease site by appropriate targeting cells would be at least as effective, and almost certainly less systemically toxic, than conventional immunotoxic therapy involving direct administation of the particular immuntoxin *per se*. Thus, with regard to autoimmune diseases, for example, clinical trials using fusion proteins composed of interleukin-2 (IL-2) (as a targeting domain) fused to a fragment of diphtheria toxin (as a toxin domain) demonstrated therapeutic efficacy in significant numbers of patients with rheumatoid arthritis and psoriasis (Moreland et al., Arthritis Rheum, 38(9):1177-1186, 1995, and Martin et al. J. Am Acad. Dermatol. 45(6):871-881, 2001, respectively; copies enclosed as Exhibit A). In addition, the efficacy of a similar immunotoxin was shown in an animal model of rheumatoid arthritis (Bacha et al. Eur. J. Immunol. 22(7):1673-1679, 1992; copy enclosed as Exhibit B). Applicants submit that use of the targeting cell approach with recombinant targeting cells secreting immunotoxins similar to those described by Moreland et al. and Martin et al. would likely, as in the studies described in the two articles, be effective in treating rheumatoid arthritis or psoriais but would result in a significantly lower level of the systemic toxicity noted in both Moreland et al. (e.g., at pages 1181-1183) and Martin et al. (e.g., at pages 876-878). Naturally, one of skill in the art would appreciate that if one were to use IL-2 as the targeting domain, it would be necessary to use targeting cells lacking surface IL-2 receptors or expressing either a low density of or low affinity surface IL-2 receptors.

With regard to infection, recent experiments in a animal model designed to test for HIV-1 replication-inhibitory agents showed that, as asserted on page 21, lines 25-30, of the instant specification, an immunotoxin containing human CD4 as a targeting domain was effective at controlling HIV-1 replication both *in vivo* and *ex vivo* (Schito et al., J. Infect Dis. 183(11):1592-

12600, 2001; copy enclosed as Exhibit C). In view of the earlier success of *in vitro* experiments using cytotoxic cells as targeting cells to deliver an immunotoxin containing a Fab fragment of an antibody specific for HIV-1 gp120 (the Yang et al. reference cited by the Examiner) to HIV-1 infected target cells, one of skill in the art would conclude from the teaching of the instant specification that using a targeting cell approach with an immunotoxin containing an appropriate non-antibody targeting domain (e.g., CD4) would be likely also be effective at controlling HIV-1 infection.

Importantly with respect to HIV-1 disease, a recent *ex vivo* study has shown that an immunotoxin containing a target cell-specific antibody (specific for the CD45RO molecule) as the targeting domain was successful at reducing the numbers of both latently and productively infected CD4+ T cells (Saavedra-Lozano et al., J. Infect. Dis. 185:306-314, 2002; copy enclosed as Exhibit D). Given the teaching of the specification, an artisan in the field would conclude that the use of non-antibody targeting domains (e.g., CD4 or a fragment of CD4 that binds to CD45RO) would be no less effective than the antibody targeting domain employed by Saavedra-Lozano et al. In addition, using a targeting cell approach would obviate to a large extent the toxic side effects of immunotoxin therapy noted in Saavedra-Lozano et al. (e.g., at page 312, column 1, paragraph 3).

If the Examiner so wishes, Applicants will supply her with copies any of a large number of references written by experts in the field expressing a high level of optimism for conventional immunotoxin treatment of a wide range of pathogenic cell diseases, e.g., those described herein as well as rejection of allogeneic and xenogeneic organ grafts. As pointed out above, approaches using targeting cells producing analagous immunotoxins would likely also be effective and certainly less systemically toxic than the conventional approaches.

In light of the comments in the Amendment and Response filed February 14, 2002, and the remarks above, Applicants respectfully submit that practicing the invention would not require more than routine experimentation by one of skill in the art and thus request withdrawal of the rejection under 35 U.S.C. §112, first paragraph, for lack of enablement.

Attached is a marked-up version of the changes being made by the current amendment.

CONCLUSION

In summary, for the reasons set forth above, Applicants maintain that all of the pending claims patentably define the invention. Applicants request that the Examiner reconsider the rejections as set forth in the Office Action and permit the pending claims to pass to allowance.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicants' undersigned representative can be reached at the telephone number listed below.

No charges are seen to be due. Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 3/27/02

Stuart Macphail, Ph.D.
Reg. No. 44,217

Fish & Richardson P.C.
45 Rockefeller Plaza, Suite 2800
New York, New York10111
Telephone: (212) 765-5070
Facsimile: (212) 258-2291

Version with markings to show changes made

In the claims:

Claims17 and 19 have been amended as follows:

17. (Amended) The targeting cell of claim 1, wherein said toxic molecule is diphtheria toxin (DT) or a functional fragment of DT.

19. (Amended) The targeting cell of claim 1, wherein said toxic molecule is: (i) a polypeptide selected from the group consisting of ricin, *Pseudomonas* exotoxin (PE), bryodin, gelonin, α-sarcin, aspergillin, restrictocin, angiogenin, Pseudomonas exotoxin, saporin, abrin, and pokeweed antiviral protein (PAP), or (ii) a functional fragment of the polypeptide of (i).

ARTHRITIS & RHEUMATISM
Vol. 38, No. 9, September 1995, pp 1177–1186

Arthritis & Rheumatism

Official Journal of the American College of Rheumatology

INTERLEUKIN-2 DIPHTHERIA FUSION PROTEIN (DAB_{486}IL-2) IN REFRACTORY RHEUMATOID ARTHRITIS

A Double-Blind, Placebo-Controlled Trial with Open-Label Extension

LARRY W. MORELAND, K. LEA SEWELL, DAVID E. TRENTHAM, R. PAT BUCY, WILLIAM F. SULLIVAN, RALPH E. SCHROHENLOHER, ROBERT H. SHMERLING, KAREN C. PARKER, WILLIAM G. SWARTZ, THASIA G. WOODWORTH, and WILLIAM J. KOOPMAN

Objective. **This pilot phase II, double-blind, placebo-controlled trial of 1 month duration, with a 2–3-month open-label extension, evaluated the safety, tolerability, biologic effects, and efficacy of interleukin-2 diphtheria fusion protein (DAB_{486}IL-2) in refractory rheumatoid arthritis (RA).**

Methods. **Forty-five RA patients were enrolled in the trial, and were randomized, after a 3–4-week disease-modifying antirheumatic drug washout, to receive a daily intravenous dose of either DAB_{486}IL-2 or placebo (saline) for 5 days. A blinded, third-party observer evaluated arthritis activity. Clinical response was defined as ≥25% improvement in swollen and tender joints and ≥25% improvement in at least 2 of 6 additional parameters. The double-blind phase was 4 weeks; placebo patients could cross over to receive open-label treatment for a maximum of 3 monthly DAB_{486}IL-2 cycles.**

Results. **In the double-blind phase, 4 of 22 patients (18%) in the treated group and none in the placebo group ($P = 0.05$) met the criteria for clinical response. During the open-label treatment phase, 11 of 36 patients (31%) and 11 of 33 patients (33%) had a clinical response after completing 2 and 3 courses of DAB_{486}IL-2, respectively. Adverse events included transient fever/chills (45%), nausea/vomiting (50%), elevated (≤3× normal) transaminases (55%), and increased joint pain (45%). Twelve patients (8 placebo, 4 DAB_{486}IL-2) did not complete 3 treatment cycles. No apparent differences were noted in CD4+ CD25+ cells of responders versus nonresponders, or of DAB_{486}IL-2–treated versus placebo-treated patients.**

Conclusion. **Clinical responses were noted in patients treated with DAB_{486}IL-2 (18%) compared with placebo (0%) in the double-blind phase. In the open-**

Portions of this study have been published in abstract form as follows: Clinical Research 41:245A, 1993; Arthritis Rheum 36 (suppl 5):R24, 1993; Arthritis Rheum 36 (suppl 9):S165, 1993; Arthritis Rheum 36 (suppl 9):S130, 1993; Arthritis Rheum 36 (suppl 5):R31, 1993, and Arthritis Rheum 36 (suppl 9):S179, 1993

Supported in part through the General Clinical Research Center of the University of Alabama at Birmingham (NIH grant RR-00032), the General Clinical Research Center at Beth Israel Hospital and Harvard Medical School (NIH grant M01-RR-01032), and by a grant from Seragen, Inc. Dr. Moreland's work was supported by an NIH Clinical Associate Physician Award. Dr. Sewell's work was supported by an NIH Physician Scientist Award. Dr. Koopman's work was supported by a Veterans Administration Merit Award.

Larry W. Moreland, MD, R. Pat Bucy, MD, PhD, William F. Sullivan, MD, Ralph E. Schrohenloher, PhD: The University of Alabama at Birmingham; K. Lea Sewell, MD, David E. Trentham, MD, Robert H. Shmerling, MD: Beth Israel Hospital and the Harvard Medical School, Charles A. Dana Research Institute and Harvard-Thorndike Laboratory, Boston, Massachusetts; Karen C. Parker, BA, William G. Swartz, MS, Thasia G. Woodworth, MD: Seragen, Inc., Hopkinton, Massachusetts; William J. Koopman, MD: The University of Alabama at Birmingham and the Birmingham Veterans Administration Hospital, Birmingham, Alabama.

Address reprint requests to Larry W. Moreland, MD, Division of Clinical Immunology/Rheumatology, SRC 068, UAB Station, Birmingham, AL 35294-7201.

Submitted for publication November 25, 1994; accepted in revised form April 8, 1995.

label phase, 33% of patients completing 3 monthly DAB_{486}IL-2 cycles had improvement in arthritis activity. Further studies of IL-2 diphtheria fusion proteins are warranted to elucidate factors that may predict clinical response and define mechanism(s) of action.

Although the etiology of rheumatoid arthritis (RA) is unknown (1,2), there is evidence that activated T lymphocytes play an important role in the pathogenesis of the disease. Evidence supporting an immunologic basis for RA includes 1) increased numbers of HLA–DR+ T cells in the peripheral blood of RA patients (3); 2) predominance of HLA–DR+ CD4+ T cells in the synovial mononuclear infiltrates of some RA patients (3,4); 3) evidence that certain major histocompatibility complex class II alleles are associated with susceptibility to RA (5–8); 4) improvement in preexisting RA in some patients who develop acquired immunodeficiency syndrome (9); and 5) improvement in some patients with refractory RA following total lymphoid irradiation (10), thoracic duct drainage (11), and treatment with cyclosporine (12).

Expression of cell-surface, high-affinity IL-2 receptors (IL-2R) characteristically accompanies the activation of T lymphocytes, a B cell subset, and monocytes (13–18). The limited distribution of IL-2R renders it an attractive target for treatment of autoimmune diseases in which activated lymphocytes are considered to play an important role. Indeed, antibodies to the p55 chain of the IL-2R have been used successfully to modify outcome in murine models of autoimmune disease such as diabetes (18), lupus nephritis (18), and collagen-induced arthritis (19). These antibodies have also been used to prevent human renal allograft rejection or to treat steroid-resistant graft-versus-host disease (20).

DAB_{486}IL-2 is a recombinant fusion protein that is cytotoxic for cells that express heterodimeric, high-affinity IL-2R (21). It is produced in *Escherichia coli* by expression of a fusion gene that contains the nucleotide sequences for the enzymatically active fragment A of diphtheria toxin (DT) and the membrane translocating portion of DT fragment B, together with the coding sequence for human IL-2 (Figure 1). At $10^{-10}M$ concentrations, DAB_{486}IL-2 binds specifically to high-affinity IL-2R, and is rapidly internalized via receptor-mediated endocytosis (22,23), whereas higher concentrations (10^{-9} to $10^{-8}M$) are required for intoxication of cells bearing intermediate affinity (p75) or single-chain, low-affinity (p55) IL-2 receptors (23). Once internalized into an acidic vesicle, the enzymatically active fragment A portion is released into the cytosol, and inhibits protein synthesis via adenosine diphosphate–ribosylation of elongation factor 2, resulting in cell death. DAB_{486}IL-2 is cytotoxic in vitro for IL-2R+–expressing human and murine tumor cells, and for phytohemagglutinin-stimulated human peripheral blood mononuclear cells (24–27). In rat adjuvant arthritis (a T cell–mediated animal model of RA), treatment of established arthritis with DAB_{486}IL-2 prevented chronic inflammation and bony erosions (28). DAB_{486}IL-2 also has been shown to suppress murine delayed hypersensitivity responses and rodent transplant rejection (29,30).

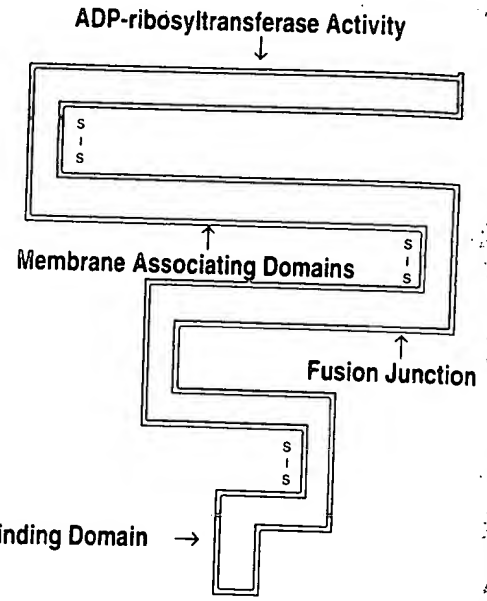


Figure 1. Structure of interleukin-2 diphtheria fusion protein (DAB_{486}IL-2). The diphtheria toxin fragment A (adenosine diphospate [ADP]–ribosyltransferase activity) and fragment B (membrane translocating portion) consist of 486 amino acids, which are synthetically fused with human IL-2 to form DAB_{486}IL-2. S—S = disulfide bonds.

In an open-label, phase I, dose-escalation study in patients with active RA that was refractory to methotrexate treatment, DAB_{486}IL-2 administration for 1 or more courses (0.07–0.1 mg/kg/day for 5–7 days) was associated with at least 25% (and often more than 50%) improvement, using the modified Paulus criteria, in about half of the patients (31). Side effects, which were transient and generally well tolerated, included nausea, transaminase elevation, fever/flu-like symptoms, and hypersensitivity, including rash and chest tightness.

The objectives of this protocol were to assess the safety, biologic effects, and efficacy of DAB_{486}IL-2 in a phase II, double-blind, placebo-controlled trial in patients with active refractory RA.

PATIENTS AND METHODS

Patients. Forty-five patients (ages 17–74) with RA that met the 1987 revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (32) were enrolled. The study was approved by the Institutional Review Boards of The University of Alabama at Birmingham (UAB) and Beth Israel Hospital. At the time of screening evaluation, all study participants exhibited active RA, as defined by the presence of: ≥9 painful/tender joints, ≥6 swollen joints, and either ≥45 minutes of morning stiffness or an erythrocyte sedimentation rate (ESR) (Westergren method) ≥28 mm/hour. In addition, all patients were without active infection and satisfied the following laboratory screening requirements: negative serum antibody test result for human immunodeficiency virus, serum creatinine ≤1.8 mg/dl, seronegative for hepatitis B antigen, and normal liver enzyme levels. HLA–DR tissue typing was obtained on all patients, using standard methods (7).

Patients were required to have failed at least 2 disease-modifying antirheumatic drug (DMARD) treatments. Concomitant treatment with stable doses (≥1 month) of a nonsteroidal antiinflammatory drug (NSAID) and/or low-dose prednisone (≤10 mg/day) was allowed. Patients were allowed to receive narcotic-containing analgesics during the DMARD washout period and throughout the study. In addition, patients were classified in either Steinbrocker functional class II or III, and Steinbrocker radiographic progression stage I, II, or III (33). Women who were pregnant or lactating were excluded, and those of childbearing potential were required to use an effective birth control method. A serum β–human chorionic gonadotropin test was performed at initial screening on all female patients of childbearing potential.

Protocol design. Following a 3–4-week run-in with DMARD washout, a second evaluation was performed to confirm eligibility and to establish baseline disease parameters. Efforts were made to assure that baseline arthritis activity parameters were stable prior to the treatment phase. If the arthritis parameters fluctuated more than 40% from the entry evaluation after the washout period, the patient was reevaluated 1 week later for level of arthritis activity; the patient qualified for randomization if the subsequent disease activity parameters had changed ≤20% from the baseline assessment.

At baseline, all qualified patients were randomized (Figure 2) using an adaptive randomization scheme stratified by age (≤50 versus >50 years) and sex, with sealed, coded envelopes held by the pharmacist at each site. The treatment (DAB_{486}IL-2 [0.075 mg/kg/day] or normal saline) was administered intravenously on days 1–5 on an outpatient basis in the General Clinical Research Center (GCRC) of each institution. The active agent was prepared in sterile, preservative-free, normal saline, and both active agent and indistinguishable placebo were administered daily as a 60-minute intravenous infusion.

Following the placebo-controlled phase, neither the physicians nor patients were informed of the treatment arm to which the patient was assigned for the first cycle. Patients not experiencing a "response" to placebo or dose-limiting adverse events were eligible to receive a total of 3 courses of DAB_{486}IL-2 at 4-week intervals.

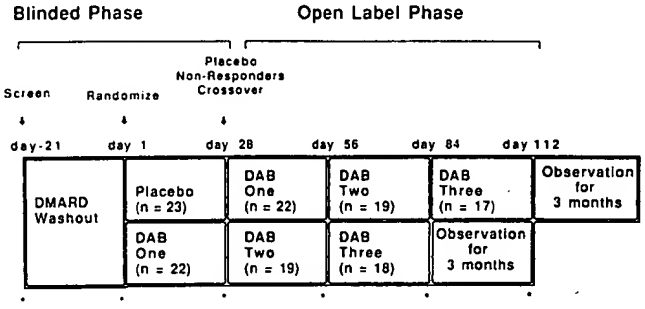


Figure 2. Overview of the design of the double-blind, placebo-controlled trial with interleukin-2 diphtheria fusion protein (DAB_{486}IL-2). If patients were receiving a disease-modifying antirheumatic drug (DMARD), this was discontinued 3 weeks before administration of DAB_{486}IL-2 or placebo. After randomization, patients received 1 infusion per day of DAB_{486}IL-2 (0.075 mg/kg/day) or placebo (saline) for 5 days. At day 28, patients were evaluated by a third-party, blinded observer for clinical efficacy; all patients were allowed to receive a total of 3 monthly courses of DAB_{486}IL-2, and arthritis activity assessment was performed after each course (*). The number of patients who received treatment at various phases of the study is listed in parentheses.

Laboratory tests. Serum chemistry studies, complete blood cell count, and platelet count were obtained at entry, baseline, before agent administration on day 1 and day 5, and weekly thereafter. Samples of peripheral blood were obtained for fluorescence-activated cell sorter (FACS) analysis of lymphocyte subsets (34) at entry, baseline, and on days 1 and 5 of each course, with a final analysis performed at completion of the third course. In a subset of patients (n = 22) enrolled at UAB, serum levels of IgM, IgG, IgA, IgM rheumatoid factor (IgMRF), and IgA-RF were measured by enzyme-linked immunosorbent assay (ELISA) (35) at baseline, and at 4 weeks following either placebo or DAB_{486}IL-2 treatment. Levels of serum IgG-RF were determined by ELISA following high performance liquid chromatography separation of the IgG fraction, as previously described (36).

Arthritis evaluation. Arthritis activity assessments were performed by the same blinded, trained assessor (1 at each GCRC) at screening, baseline, and at 4 weeks after the agent administration. The arthritis activity assessments included determination of the number of swollen (66 maximum) and painful (68 maximum) joints, duration of morning stiffness, grip strength (mean of 3 repetitions using a hand dynamometer), time interval to walk 50 feet, and patient and observer global assessments (1 = very good, 2 = good, 3 = fair, 4 = poor, 5 = very poor). The blinded observers at each GCRC performed arthritis activity assessments, but did not participate in any other aspect of the trial. Prior to initiation of the study, the blinded assessors from both institutions met in a 1-day training session to examine the same panel of patients and reach a consensus on joint scoring.

A clinically meaningful response was defined according to a modified Paulus approach (37), requiring that the following criteria be met: ≥25% improvement (from baseline) in number of painful and swollen joints and at least 2 of the following 6 criteria: ≥25% improvement in walk time, grip strength, duration of morning stiffness, ESR, and improvement either by 2 integers or from grade 2 to 1 in the patient and/or blinded observer assessments.

Table 1. Demographic and clinical characteristics of 45 rheumatoid arthritis (RA) patients enrolled in the study of interleukin-2 diphtheria fusion protein ($DAB_{486}IL-2$) treatment

Characteristics	Treatment group	
	Placebo (n = 23)	$DAB_{486}IL-2$ (n = 22)
Age (years)		
Mean ± SD	50 ± 12	52 ± 14
Range	24–67	17–74
Ratio of men to women	8:15	6:16
Disease duration (years)		
Mean ± SD	9 ± 6	10 ± 7
Range	2–23	2–26
Mean no. of prior therapies*	4.5	4.7
No. of patients receiving prednisone (≤10.0 mg/day)	19†	11†
Mean prednisone dose (mg/day)	8.1	6.1
No. of patients receiving NSAIDs‡	18	17

* Therapies are listed in Table 2.
† $P = 0.05$.
‡ NSAIDs = nonsteroidal antiinflammatory drugs.

Preparation of $DAB_{486}IL-2$. $DAB_{486}IL-2$ was supplied as a sterile frozen (≤−15°C) solution formulated in Tris buffered saline. Trace amounts of horse IgG, protamine sulfate, and *E coli* proteins may have been present in the final formulation as process residuals.

Immune response to $DAB_{486}IL-2$ and IL-2. Levels of antibody to DT, IL-2, $DAB_{486}IL-2$, horse IgG, and *E coli* were assessed at baseline and at 4 weeks posttreatment, according to methods previously described (27). The antibody titer was determined as the highest dilution of serum that produced an absorbance at 405 nm of ≥0.1 when analyzed with an automated plate reader.

Immunophenotypic analysis of peripheral blood lymphocytes. Immunophenotyping of patient samples was performed by the whole blood lysis method, as previously described (34). IL-2R was determined on both CD4 and CD8 subsets of T cells using phycoerythrin-conjugated, anti–IL-R monoclonal antibody (Becton Dickinson, Mountain View, CA). Conjugate, isotype-control monoclonal antibodies were used to establish the marker placement to determine CD25+ cells.

Serum soluble IL-2R. Soluble IL-2R (sIL-2R) levels were determined using methods previously described (27). The concentration of sIL-2R in each sample was determined from the standard curve. The lower limit of detection of sIL-2R was 20 p*M*.

Statistical analysis. Patient demographic information is presented by initial treatment group. Descriptive statistics are provided in the form of means, standard deviations, and ranges for all continuous variables, and frequencies and percentages for all categorical variables. Inferential statistics are provided as well; 2-sample *t*-tests were used to compare treatment groups with respect to age, sex, disease duration, number of prior DMARD therapies, prednisone use, immunoglobulin and RF levels, and to ensure comparability of investigational sites with respect to all baseline demographic variables.

Data on adverse events are presented by initial treatment group for the blinded phase of the study, and by active course number for the subsequent open-label phase. Fisher's exact test was used to compare treatment groups with respect to the incidence of individual adverse events.

Response data are summarized by initial treatment group and course number with means and standard deviations. Repeated-measures analyses were used to compare the 2 treatment groups with respect to the changes in mean response levels from baseline to post–course 1 (blinded-treatment phase) and, additionally, with respect to the changes in mean levels of response from baseline through all active courses of the study drug (open-label phase).

FACS data were analyzed using a repeated-measures design to test for differences between treatment groups in the percentage of lymphocytes expressing various cell-surface markers over time. This analysis method permitted the testing of a time effect for each lymphocyte subpopulation, as well as an interaction effect between time and treatment group.

RESULTS

Demographic and clinical characteristics. The demographic and clinical characteristics of the 45 RA patients enrolled in this study are presented in Table 1. There were no significant differences between the treatment groups with regard to age, sex, disease duration, or number of prior DMARDs. Nineteen placebo patients (83%) were receiving prednisone (mean 8.1 mg/day) at entry, compared with 11 $DAB_{486}IL-2$–treated patients (52%) (mean 6.1 mg/day). The difference between the treatment groups in use of prednisone was statistically significant ($P = 0.05$). All patients had been treated unsuccessfully with at least 2 DMARDs, and the mean number of prior treatments was 4.5. All placebo patients and 95% of the $DAB_{486}IL-2$ patients had received methotrexate prior to study enrollment. The DMARDs that were discontinued during the 3-week run-in period are summarized in Table 2. Eighteen patients in the placebo

Table 2. Disease-modifying antirheumatic drugs (DMARDs) discontinued at screening visit in 45 RA patients enrolled in the study of $DAB_{486}IL-2$*

DMARD	Treatment group	
	Placebo, no. (%)	$DAB_{486}IL-2$, no. (%)
Methotrexate	8 (35)	5 (23)
Methotrexate, azathioprine	0 (0)	1 (5)
Methotrexate, sulfasalazine, hydroxychloroquine	1 (4)	0 (0)
Methotrexate, D-penicillamine, hydroxychloroquine	0 (0)	1 (5)
Gold salts (parenteral)	1 (4)	0 (0)
Hydroxychloroquine	0 (0)	2 (9)
Sulfasalazine	1 (5)	0 (0)
Azathioprine	2 (9)	1 (5)
Azathioprine, hydroxychloroquine	2 (9)	0 (0)
None	8 (35)	12 (55)

* See Table 1 for other definitions.

Table 3. Clinical responses in RA patients after completing up to 3 courses of DAB_{486}IL-2 treatment*

DAB_{486}IL-2 course	No. of patients completing the course	Responders randomized initially to DAB_{486}IL-2 arm†	Responders randomized initially to placebo arm†	Total no. (%) of responders after receiving DAB_{486}IL-2†
First	42	4	3	7 (17)
Second	36	7	4	11 (31)
Third	33	6	5	11 (33)

* See Table 1 for definitions.
† See Patients and Methods for definition of clinical response.

group and 17 in the DAB_{486}IL-2–treated group continued to receive NSAIDs throughout the study.

Clinical effect of DAB_{486}IL-2. *Blinded control phase.* The mean ± SD number of tender and swollen joints at baseline was 28.3 ± 13.2 and 23.0 ± 14.8 in the placebo group, and 34.0 ± 15.3 and 26.4 ± 17.3 in the DAB_{486}IL-2 group, respectively. Based on the response criteria used in this study, 4 of 22 treated patients (18%) and none of the placebo patients (0 of 23) responded at 4 weeks after the first course (P = 0.05). A total of 7 of 42 patients (17%) (including those who initially received placebo and later open-label DAB_{486}IL-2) met response criteria after completing 1 course of DAB_{486}IL-2. Both blinded observer and patient global assessments (data not shown) were statistically significant (P = 0.002 and P = 0.0001, respectively) for DAB_{486}IL-2 compared with placebo. No significant changes in ESR were observed over time. As illustrated in Figure 2, 1 placebo patient and 3 DAB_{486}IL-2–treated patients did not enter the open-label phase (see below for reasons for withdrawal).

Open-label phase. In the open-label treatment phase, 11 of 36 patients (31%) and 11 of 33 patients (33%) responded after completing 2 and 3 courses of DAB_{486}IL-2, respectively (Table 3). The 11 patients exhibiting clinical improvement ("responders") showed ≥60% improvement in the number of swollen and painful joints, morning stiffness, and grip strength. HLA–DR typing revealed 7 of 11 responders and 15 of 33 nonresponders to have a DR allele reported to be associated with more severe disease (0401, 0404, 0405) (38).

Safety of DAB_{486}IL-2. The adverse events recorded during the study period are summarized in Tables 4 and 5. Side effects that typically occurred on the first day of DAB_{486}IL-2 treatment included chills/fever (45%), nausea (50%), and increased joint pain (45%). In the blinded phase, there was a statistically significant increase in the incidence of chills/fever and nausea in the patients treated with DAB_{486}IL-2 compared with those treated with placebo. These symptoms were usually self-limited and responded to treatment with acetaminophen, H_2 antagonists, and prochlorperazine. High fever led to hospitalization and treatment with intravenous methylprednisolone during the second infusion in a 73-year-old woman. Serum glutamic oxaloacetic transaminase and glutamic pyruvate transaminase elevations (≤3× upper limits of normal) occurred in 55% of the DAB_{486}IL-2–treated patients during the blinded course and returned to normal within 3 weeks. The difference in the incidence of transaminase elevations between the group of patients treated with DAB_{486}IL-2 and the group treated with placebo was statistically significant. In the open-label phase, second and third courses with DAB_{486}IL-2 were associated with less frequent transaminase elevations (Table 5). There were no clinically significant changes noted in renal function or hematologic parameters during the entire study.

Twelve of the 45 patients withdrew from the study. Figure 2 shows the number of patients who started each treatment phase of the study. Three patients withdrew from the study secondary to ad-

Table 4. Adverse events reported in RA patients during double-blind phase of the study (up to 4 weeks after receiving DAB_{486}IL-2 treatment)*

Adverse event	Treatment group: Placebo (n = 23)	Treatment group: DAB_{486}IL-2 (n = 22)
Elevated transaminase (≤3× normal)	0	12†
Nausea/vomiting	3	11†
Increased joint pain	9	10
Chills/fever	3	10†
General weakness	3	4
Paresthesias	4	3
Headache	4	3
Diarrhea	2	3
Thrombocytosis (>450,000/mm^3)	2	6
Anorexia	0	3
Dizziness	2	2

* See Table 1 for definitions.
† $P \leq 0.05$ versus placebo.

Table 5. Adverse events reported in RA patients during entire study (up to 4 weeks after receiving last treatment with $DAB_{486}IL\text{-}2$)*

Adverse event	First course (n = 44)†	Second course (n = 38)†	Third course (n = 35)†
Elevated transaminase (≤3× normal)	23	4	1
Nausea/vomiting	18	16	10
Increased joint pain	21	16	13
Chills/fever	14	20	13
Headache	4	6	4
General weakness	8	3	2
Dizziness	4	5	0
Paresthesias	6	7	7
Diarrhea	4	3	4
Anorexia	6	5	2
Back pain (hypersensitivity reaction)	0	0	1
Hypotension	0	1	0

* See Table 1 for definitions.
† Numbers reflect patients who started a course.

verse events. One withdrew after requiring hospitalization for symptomatic hypotension (blood pressure of 60/40 mm Hg), which occurred 30 minutes after receiving the second course of $DAB_{486}IL\text{-}2$ and responded to intravenous fluids. A second patient withdrew because of back pain experienced during infusion with $DAB_{486}IL\text{-}2$ (third $DAB_{486}IL\text{-}2$ course), which responded to intravenous diphenylhydramine hydrochloride and was believed to be a hypersensitivity reaction. A third patient, a 66-year-old woman, developed a fatal myocardial infarction 7 days after receiving the first course of $DAB_{486}IL\text{-}2$. She had no previous history of heart disease; however, she had multiple risk factors for coronary atherosclerosis, including hypertension, family history of myocardial infarction, hyperlipidemia, glucose intolerance, and history of smoking. An autopsy was not performed.

Six subjects withdrew because of lack of efficacy or increased joint pain (3 initially randomized to the placebo group and 3 randomized to the $DAB_{486}IL\text{-}2$ group). One patient withdrew because of severe worsening of joint pain and the occurrence of abdominal pain during treatment with $DAB_{486}IL\text{-}2$ (first course).

Eight patients initially randomized to the placebo group dropped out at various time points; 1 patient withdrew during the blinded phase, 3 patients withdrew following the first $DAB_{486}IL\text{-}2$ course (2 prior to the week-4 assessment), 2 patients withdrew after receiving the second $DAB_{486}IL\text{-}2$ course (1 prior to the week-4 assessment), and 2 patients withdrew prior to the week-4 assessment after the third $DAB_{486}IL\text{-}2$ course. The reasons for withdrawal included lack of improvement (3 patients), adverse experiences (3 patients), lost to followup (1 patient), and 1 patient self-withdrawal.

No serious infections occurred during the study. The infections that did occur were considered to be mild and not likely related to the study drug; these included urinary tract infections, herpes labialis,

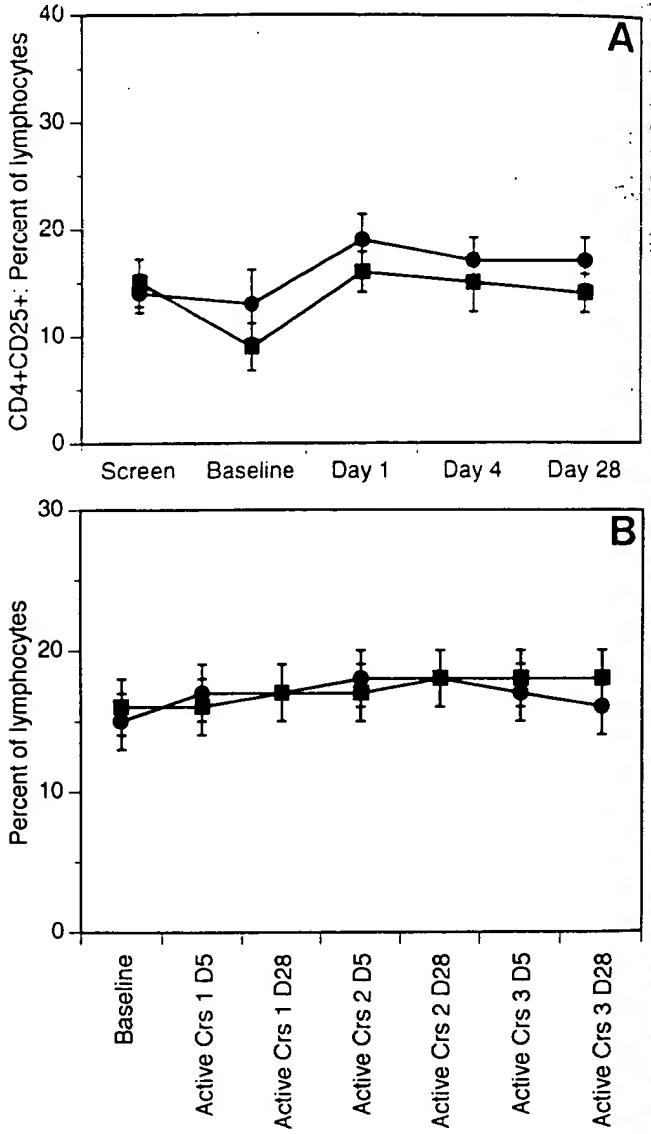


Figure 3. A, Percentage (mean ± SEM) of CD4+CD25+ lymphocytes in the interleukin-2 diphtheria fusion protein ($DAB_{486}IL\text{-}2$)–treated (■) versus placebo (●) groups during the double-blind portion of the study. B, Percentage (mean ± SEM) of CD4+ T cells positive for CD25 (IL-2 receptor) at baseline and during treatment phases in those patients who were nonresponders (●) and responders (■) to $DAB_{486}IL\text{-}2$ treatment, according to the 25% Paulus criteria (see text for description). Crs = course; D = posttreatment day.

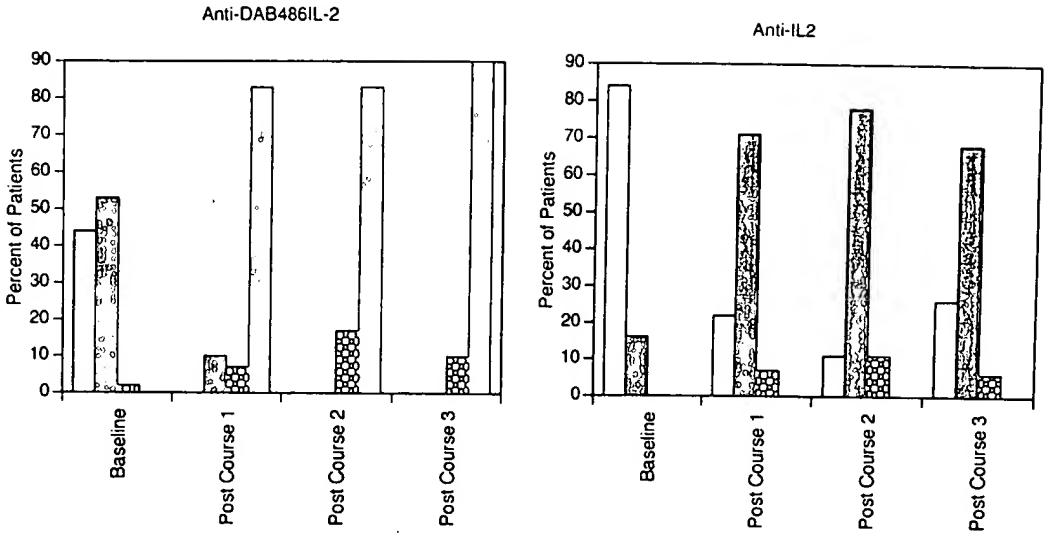


Figure 4. Percentage of patients with pretreatment and posttreatment antibodies to diphtheria toxin and IL-2. Open bars = rank <1–1 (titer ≤1:5); shaded bars = rank 2–3 (titer 1:25–1:125); checked bars = rank 4–5 (titer 1:625–1:3,125); black bars = rank ≥6 (titer ≥1:15,625) (see Patients and Methods for determination of antibody levels and Results for definitions of rank titers). Anti-DAB486IL-2 = anti–interleukin-2 diphtheria fusion protein.

vaginal yeast infection, upper respiratory infection, and tinea corporis.

Laboratory studies. *Lymphocyte surface markers.* No significant differences were observed between the active drug– and placebo-treated patients during the double-blind period, either in the percentage (or absolute numbers) of lymphocytes expressing CD4 or CD8, or in the percentage coexpressing CD8 and CD25, or CD4 and CD25 (Figure 3A). No apparent differences were observed in CD25+ CD4+ lymphocytes in the responders when compared with nonresponders (Figure 3B). Although the placebo patients had a higher mean percentage of lymphocytes expressing CD25 over all time points compared with the active treatment group, this difference was not statistically significant (Figure 3A).

Soluble IL-2R levels. All placebo patients and 95% of patients randomized to initially receive DAB_{486}IL-2 exhibited slightly elevated (≥50 p*M*) sIL-2R levels at entry. This difference was not statistically significant. No apparent changes from baseline were noted in either the placebo or DAB_{486}IL-2 groups at the end of the blinded phase or after subsequent courses with DAB_{486}IL-2 (data not shown). The level of sIL-2R did not correlate at any time with disease activity, as measured by swollen and painful/tender joint counts.

Antibody response to DT, IL-2, and E coli. Figure 4 depicts the levels of antibodies to DT and IL-2, pre-study and post-study. Rank titers are defined as ≤1 (not significant), 2–3 (low titer), 4–5 (moderate titer), and ≥6 (high titer). Anti–IL-2 antibodies measured pretreatment included titers of <1:5 in 52% of patients, 1:5 in 32%, and 1:25 in the remaining 16% of patients. At the end of the study, anti–IL-2 antibody titers generally increased, as indicated by the fact that 76% of patients had titers between 1:25 and 1:625.

At pre-study, anti-DT antibodies were present in the following titers: 7% of patients had titers <1:5, 32% had a level of 1:5, and the remaining 61% had titers of 1:25 to 1:625. At the end of the study, the levels of anti-DT antibodies were much higher: 9% of patients had titers of 1:3,125, and the remaining 91% had titers ≥1:15,625. Serum antibodies to DT and IL-2 reached their highest levels after the first treatment and did not increase with the second and third DAB_{486}IL-2 treatments. Antibody titers did not predict hypersensitivity reactions.

The possibility that the presence of anti-DT antibodies might influence responses to DAB_{486}IL-2 was also examined. In this regard, 11 of 15 responders (73%) had anti-DT titers ≥1:25 at study entry. These levels were similar to the 61% prevalence of titers ≥1:25 in the entire study group at entry.

Most patients (95%) had significant levels of antibody to *E coli* pre-study; however, only 2 patients exhibited a significant (≥2 ranks) increase in titer after DAB_{486}IL-2 administration.

Immunoglobulin and rheumatoid factor levels. In 22 patients studied at UAB, levels of IgM, IgM-RF, and IgA increased significantly after 4 weeks in placebo patients compared with baseline values, whereas

no significant changes were noted in the DAB_{486}IL-2–treated group (data not shown). No differences were observed in either group in IgG, IgG-RF, and IgA-RF levels over time.

DISCUSSION

We report a pilot phase II trial using a recombinant IL-2R–specific cytotoxin (DAB_{486}IL-2) for the treatment of refractory RA. The refractory nature of disease in these patients is exemplified by the fact that most had failed 4 or more DMARD treatments, and all but 2 patients had failed treatment with methotrexate. These patients had longstanding disease (mean duration 9 and 10 years in the 2 treatment groups), which may be the least likely to respond to agents directed against activated lymphocytes (2).

During the placebo-controlled period, 18% of patients in the DAB_{486}IL-2 group, but no patients in the placebo group, exhibited significant clinical improvement based on a response definition requiring ≥25% improvement in multiple disease activity parameters. Of the 33 patients who completed 3 courses of DAB_{486}IL-2, 33% met clinical response criteria. This result reflects 24% of the original study participants. The response rates observed in this study compare favorably with those reported in the phase I study using DAB_{486}IL-2 (31). We chose a conservative, mid-dose level for this trial due to the maximally tolerated doses noted in the phase I trial (31). Retrospective analysis of HLA–DR typing did not identify patients likely to respond, as there was no disproportionate distribution of DR types associated with more aggressive disease in the nonresponder group (38). Further studies are needed to fully assess the significance of the reported DR types associated with severe RA. Recently reported data from another large clinical trial also failed to detect an association between the "rheumatoid" epitope copy-number and disease severity (39).

The study design, with a 3–4-week DMARD washout and unchanged NSAID and prednisone doses, was intended to establish a reasonably stable level of arthritis activity before initiating treatment. When DMARDs are discontinued in experimental treatment trials, a concern arises as to whether the DMARD antiarthritic effect has been completely removed with a short washout (as with hydroxychloroquine or gold salts), and whether a significant arthritis flare will occur when shorter-acting DMARDs (e.g., methotrexate) are discontinued. To minimize this latter concern, we included strict criteria for baseline arthritis variability, specifically the 40% maximum permitted fluctuation in arthritis parameters over the 3-week washout period. We have also evaluated the time to flare in RA patients withdrawing from methotrexate therapy in this and 2 other DAB_{486}IL-2 trials, and did not confirm a significantly greater increase in arthritis activity after a 3–4-week washout when discontinuing the methotrexate compared with "longer-acting" DMARDs (40). In these patients, a >20% increase in swollen joint counts occurred in 37% of patients receiving methotrexate, whereas 45% of patients being treated with other DMARDs had similar increases in joint swelling after 4 weeks of DMARD washout (40).

The use of blinded third-party evaluators and the above-mentioned efforts to assure stable levels of disease activity prior to treatment are 2 significant strengths of this placebo-controlled trial. In addition, efforts were made to minimize the need for unblinding due to the occurrence of fever and other side effects, by administration of acetaminophen, 650 mg every 6 hours during the first treatment week. Nevertheless, those persons receiving DAB_{486}IL-2 treatment had a higher incidence of fever and chills compared with the placebo group (Table 4), and these events could have potentially unblinded patients to their initial treatment.

Previous in vitro and in vivo studies have suggested that DAB_{486}IL-2 preferentially targets activated (IL-2R+) lymphocytes (24–30). To address whether this might be the mechanism of action of DAB_{486}IL-2 in RA, several biologic parameters were evaluated, including immunophenotypic analysis of peripheral blood lymphocytes by FACS, serial measurement of serum sIL-2R levels, and assessment of in vivo expression of immunoglobulin and RF isotypes.

As determined by FACS analysis, there was no statistically significant difference between the placebo and DAB_{486}IL-2 groups with regard to percentage (Figure 3A) or absolute numbers of lymphocytes expressing CD25 and CD4 at the end of the blinded phase. Furthermore, no apparent differences were observed in CD25+ CD4+ lymphocytes in the responders when compared with nonresponders. We speculated that a subpopulation of high-density IL-2R+ cells might be preferentially targeted by the fusion protein. Nevertheless, no apparent differences were observed in the frequency of high-density CD25+ lymphocytes (CD4+ or CD8+) in DAB_{486}IL-2– or placebo-treated patients, or in responders compared with nonresponders. Although we were not able to detect statistically significant changes in CD25+ cells, this may relate to the insensitivity of the labora-

tory evaluation to detect transient small changes, the small number of patients assessed, the existence of a population of CD25+ cells preferentially targeted by treatment with DAB_{486}IL-2, or effects locally present in the joints that were not detected in the circulating lymphocyte population. Furthermore, there was no significant difference in serum sIL-2R levels between placebo- and DAB_{486}IL-2–treated patients at study entry. It is conceivable that the clinical effects of DAB_{486}IL-2 fusion protein derive from IL-2R–independent mechanisms.

Although one DAB_{486}IL-2 treatment course did not result in decreased immunoglobulin or RF levels over a 4-week period, increases in IgM-RF, IgM, and IgA were observed in the control group. Parallel observations demonstrated that DAB_{486}IL-2 treatment resulted in decreased in vitro production of IgM and IgM-RF by peripheral blood B lymphocytes compared with placebo controls; this raises the possibility that DAB_{486}IL-2 may diminish B cell function either directly or indirectly through effects on T helper cell function (41).

Symptomatic toxicities in this trial were common; nausea/emesis and fever occurred in approximately half of the patients initially treated with DAB_{486}IL-2. The gastrointestinal side effects and fever/chills were transient and usually resolved within hours; however, some patients required intravenous medications or fluid administration. The only significant laboratory abnormalities were transaminase elevations; these were transient and returned to pretreatment levels within 2–4 weeks. No cumulative or persistent liver enzyme abnormalities were noted, and transaminases were less likely to rise with repeat courses. The side effect profile seen in this trial is similar to that noted in the phase I trial (31). Three patients withdrew from the study for significant adverse events, which included hypotension, hypersensitivity reaction, and increased joint pain. No opportunistic infections occurred.

A potential concern with using this agent would be the development of neutralizing antibodies to IL-2. All patients developed anti-DT antibodies after receiving 3 monthly courses of DAB_{486}IL-2, and only 1 patient developed symptoms suggestive of a hypersensitivity reaction. There was no correlation between the presence of anti-DT antibodies before treatment with DAB_{486}IL-2 and subsequent development of elevated hepatic transaminase levels. Most importantly, there was no association between pretreatment or posttreatment anti-DT antibodies and clinical response to DAB_{486}IL-2. It is likely that antibodies to DT do not impair the target cell cytotoxicity, since anti-DT antibodies bind to the DT portion without affecting the binding of the human IL-2R ligand of DAB_{486}IL-2. Indeed, in the adjuvant arthritis model, rats preimmunized with DT developed antibodies to DT, but these antibodies did not alter the therapeutic efficacy of DAB_{486}IL-2 (28).

In conclusion, this double-blind, placebo-controlled study demonstrates that some patients with refractory RA experience clinical improvement after receiving 1 or more 5-day courses of DAB_{486}IL-2. Although these patients required monitoring for potential infusion-related symptoms, symptoms were relieved by medication, and all adverse events were reversible. Further studies are needed to characterize which patients may respond to DAB_{486}IL-2, and to elucidate the precise mechanism(s) of action.

ACKNOWLEDGMENTS

The outstanding assistance of the clinical coordinators, Tina Parkhill and Julie Carpenter, is much appreciated, as is the technical expertise of Betsy Perry in the preparation of the manuscript, and the expertise of Janie Feldman in preparation of the immunophenotypic analysis. In addition, we appreciate the support of the faculty and fellows in the Division of Clinical Immunology and Rheumatology at UAB, of Fred Kantrowitz, MD, for blinded assessments at Beth Israel Hospital, and the expert nursing assistance at both UAB and Beth Israel Hospital. Finally, we acknowledge Drs. Gerald and Barbara Nepom at the Virginia Mason Research Center (Seattle, Washington) for performing the HLA typing.

REFERENCES

1. Harris ED Jr: Rheumatoid arthritis: pathophysiology and implications for therapy. N Engl J Med 322:1277–1289, 1990
2. Koopman WJ, Gay S: Do nonimmunologically mediated pathways play a role in the pathogenesis of rheumatoid arthritis? Rheum Dis Clin North Am 19:107–122, 1993
3. Burmester GR, Yu DTY, Irani AM, Kunkel HG, Winchester RJ: Ia+ T cells in synovial fluid and tissues of patients with rheumatoid arthritis. Arthritis Rheum 24:1370–1376, 1981
4. Van Boxel JA, Paget SA: Predominantly T-cell infiltrate in rheumatoid synovial membrane. N Engl J Med 293:517–520, 1975
5. Stastny P: Association of the B-cell alloantigen DRw4 with rheumatoid arthritis. N Engl J Med 298:869–871, 1978
6. Schiff B, Mizrachi Y, Orgad S, Yaron M, Gazit E: Association of HLA-Aw31 and HLA-DR1 with adult rheumatoid arthritis. Ann Rheum Dis 41:403–404, 1982
7. Gregersen PK, Silver J, Winchester RJ: The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 30:1205–1213, 1987
8. Nepom GT, Byers P, Seyfried C, Healey LA, Wilske KR, Stage D, Nepom BS: HLA genes associated with rheumatoid arthritis: identification of susceptibility alleles using specific oligonucleotide probes. Arthritis Rheum 32:15–21, 1989
9. Calabrese LH, Wilske WS, Perkins AD, Tubbs RR: Rheumatoid arthritis complicated by infection with the human immuno-

deficiency virus and the development of Sjögren's syndrome. Arthritis Rheum 32:1453–1457, 1989
10. Tanay A, Field EH, Hoppe RT, Strober S: Long-term followup of rheumatoid arthritis patients treated with total lymphoid irradiation. Arthritis Rheum 30:1–10, 1987
11. Paulus HE, Machleder HI, Levine S, Yu DTY, MacDonald NS: Lymphocyte involvement in rheumatoid arthritis: studies during thoracic duct drainage. Arthritis Rheum 20:1249–1262, 1977
12. Yocum DE, Klippel JH, Wilder RL, Gerber NL, Austin HA, Wahl SM, Lesko L, Minor JR, Preuss HG, Yarboro C: Cyclosporin A in severe, treatment-refractory rheumatoid arthritis: a randomized study. Ann Intern Med 109:863–869, 1988
13. Smith KA: Interleukin-2: inception, impact and implications. Science 240:1169–1176, 1988
14. Waldman TA, Goldman CK, Robb RJ, Depper JM, Leonard WJ, Sharrow SO, Bongiovanni KF, Korsmeyer SJ, Greene WC: Expression of interleukin-2 receptors on activated human B-cells. J Exp Med 160:1450–1466, 1984
15. Holter W, Goldman CK, Casabo L, Nelson DL, Green WC, Waldmann TA: Expression of functional IL-2 receptors by lipopolysaccharide and interferon-γ stimulated monocytes. J Immunol 138:2917–2922, 1987
16. Waldmann TA: The interleukin-2 receptor. J Biol Chem 266: 2681–2684, 1991
17. Rubin LA, Kurman CC, Fritz ME, Biddison WE, Boutin B, Yarchoan R, Nelson DL: Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. J Immunol 135:3172–3177, 1985
18. Kelley VE, Gaulton GN, Hattori M, Ikegami H, Eisenbarth G, Strom TB: Anti-interleukin-2 receptor antibody suppresses murine diabetic insulitis and lupus nephritis. J Immunol 140:59–61, 1988
19. Banerjee S, Wei B, Hillman K, Luthra HS, David CS: Immunosuppression of collagen-induced arthritis in mice with an anti-IL-2 receptor antibody. J Immunol 141:1150–1154, 1988
20. Peyronnet P, LeMauff B, Hourmaut M, Cantarovich D, Dubigeon P, Olive P, Mawas C, Delaage M, Hirn J, Jacques Y, Soulillou JP: Prophylactic treatment of human kidney allograft recipients with a monoclonal antibody (33B3.1) directed against interleukin 2 receptor. Transplant Proc 20:300–302, 1988
21. Williams D, Parker K, Bacha P, Bishai W, Genbauffe, Strom TB, Murphy JR: Diphtheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphtheria toxin-related interleukin-2 fusion protein. Protein Eng 1:493–498, 1987
22. Walz G, Zanker B, Brand K, Waters C, Genbauffe F, Zeldis JB, Murphy JR, Strom TB: Sequential effects of interleukin 2-diphtheria toxin fusion protein on T-cell activation. Proc Natl Acad Sci U S A 86:9485–9488, 1989
23. Williams DP, Snider CE, Strom TB, Murphy JR: Structure/function analysis of interleukin-2 toxin (DAB486IL-2). J Biol Chem 265:11885–11889, 1990
24. Bacha P, Williams DP, Waters C, Williams JM, Murphy JR, Strom TB: Interleukin-2 receptor-targeted cytotoxicity: interleukin-2 receptor mediated action of a diphtheria toxin related interleukin-2 fusion protein. J Exp Med 167:612–622, 1988
25. Waters CA, Schimke PA, Snider CE, Itoh K, Smith KA, Nichols JC, Strom TB, Murphy JR: Interleukin-2 receptor targeted cytotoxicity: receptor binding requirements for entry of a diphtheria toxin-related interleukin 2 fusion protein into cells. Eur J Immunol 20:785–791, 1990
26. Kiyokawa T, Shirono K, Hattori T, Nishimura H, Yamaguchi K, Nichols JC, Strom TB, Murphy JR, Takatsuki K: Cytotoxicity of interleukin-2 toxin toward lymphocytes from patients with adult T-cell leukemia. Cancer Res 49:4042–4046, 1989
27. LeMaistre CF, Meneghetti C, Rosenblum M, Reuben J, Parker K, Shaw J, Deisseroth A, Woodworth T, Parkinson DR: Phase I trial of an interleukin-2 fusion toxin (DAB486IL-2) in hematologic malignancies expressing the IL-2 receptor. Blood 79:2547–2554, 1992
28. Bacha P, Forte SE, Perper SJ, Trentham DE, Nichols JL: Anti-arthritic effect demonstrated by an interleukin-2 receptor targeted cytotoxin (DAB486IL-2) in rat adjuvant arthritis. Eur J Immunol 22:1673–1679, 1992
29. Kelley VE, Bacha P, Pankewycz O, Nichols JC, Murphy JR, Strom TB: Interleukin-2 diphtheria toxin fusion protein can abolish cell-mediated immunity in vivo. Proc Natl Acad Sci U S A 85:3980–3984, 1988
30. Kirkman RL, Bacha P, Barrett LV, Forte S, Murphy JR, Strom TB: Prolongation of cardiac allograft survival in murine recipients treated with a diphtheria toxin related interleukin-2 fusion protein. Transplantation 47:327–330, 1989
31. Sewell KL, Parker KC, Woodworth TG, Reuben J, Swartz W, Trentham DE: DAB_{486}IL-2 fusion toxin in refractory rheumatoid arthritis. Arthritis Rheum 36:1223–1233, 1993
32. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA Jr, Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, Hunder GG: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324, 1988
33. Steinbrocker O, Traeger CH, Batterman RC: Therapeutic criteria in rheumatoid arthritis. JAMA 140:659–662, 1949
34. Moreland LW, Bucy RP, Tilden A, Pratt PW, LoBuglio AF, Khazaeli M, Everson MP, Daddona P, Ghrayeb J, Kilgarriff C, Sanders ME, Koopman WJ: Use of a chimeric monoclonal anti-CD4 antibody in patients with refractory rheumatoid arthritis. Arthritis Rheum 36:307–318, 1993
35. Tomana M, Schrohenloher RE, Koopman WJ, Alarcón GS, Paul WA: Abnormal glycosylation of serum IgG from patients with chronic inflammatory diseases. Arthritis Rheum 31:333–338, 1988
36. Schrohenloher RE, Koopman WJ: Fractionation of serum by high performance liquid chromatography (HPLC) facilitates analysis of IgG-RF (abstract). Arthritis Rheum (suppl 6) 36:R22, 1994
37. Paulus HE, Egger MJ, Ward JR, Williams HJ, and the Cooperative Systematic Studies of Rheumatic Diseases Group: Analysis of improvement in individual rheumatoid arthritis patients treated with disease-modifying antirheumatic drugs, based on the findings in patients treated with placebo. Arthritis Rheum 33:477–484, 1990
38. Weyand CM, Xie C, Goronzy JJ: Homozygosity for the HLA-DRB1 allele selects for extraarticular manifestations in rheumatoid arthritis. J Clin Invest 89:2033–2039, 1992
39. Reveille JD, Alarcón GS, Fowler S, Pillemer S, Neuner R, Clegg DO, Trentham D, Leisen J, Cooper S, Duncan H, Mikhail I, Tuttleman M, Heyse S, Tilley B: Lack of a gene-dose effect for the "rheumatoid" epitope in RA patients from a large multicenter clinical trial (abstract). Arthritis Rheum 37 (suppl 9): S359, 1994
40. Furst DE, Parker K, Moreland LW, Woodworth T: Variations in swollen joint counts during DMARD wash-outs and placebo treatment in patients entering DAB-IL-2 trials (abstract). Arthritis Rheum 36 (suppl 9):S179, 1993
41. Schrohenloher RE, Koopman WJ, Woodworth TG, Moreland LW: Suppression of in vitro IgM and IgM rheumatoid factor production by DAB_{486}IL-2 fusion toxin therapy in rheumatoid arthritis (abstract). Arthritis Rheum 36 (suppl 9):S130, 1993

A multicenter dose-escalation trial with denileukin diftitox (ONTAK, DAB_{389}IL-2) in patients with severe psoriasis

Ann Martin, MD,[a] Elsa Gutierrez, MD,[a] Jennie Muglia, MD,[b] Charles J. McDonald, MD,[b] Cynthia Guzzo, MD,[c] Alice Gottlieb, MD, PhD,[d] Amy Pappert, MD,[d] W. Thomas Garland, MD,[e] Jerry Bagel, MD,[f] and Patricia Bacha, PhD[g] *St Louis, Missouri; Providence, Rhode Island; Philadelphia, Pennsylvania; New Brunswick, Lawrenceville, and East Windsor, New Jersey; and San Diego, California*

Background: Denileukin diftitox, a fusion protein targeting both malignant and normal activated lymphocytes, has been shown previously to have antipsoriatic activity. However, the ideal dosing regimen for treating psoriasis was not established.

Objective: We examined the safety and efficacy of denileukin diftitox in patients with severe plaque-type psoriasis.

Methods: This was a cohort dose-escalation trial. Patients were administered denileukin diftitox on 3 consecutive days every other week. Patients were evaluated for toxicity, improvement in psoriasis, immunogenicity, and serum levels.

Results: Thirty-five patients were treated at 3 dose levels. Eight patients had a 50% decrease or more in Psoriasis Area and Severity Index score from baseline (0/10 at 0.5 μg/kg per day, 1/10 at 1.5 μg/kg per day, and 7/15 at 5 μg/kg per day). Adverse events primarily consisted of constitutional events and skin reactions.

Conclusions: The potential antipsoriatic activity of denileukin diftitox demonstrated in this study was comparable to that observed in other psoriasis studies with this agent. However, this dosing regimen was better tolerated than the dosing regimen used in the last study with denileukin diftitox in psoriasis patients. (J Am Acad Dermatol 2001;45:871-81.)

Psoriasis is characterized by accelerated proliferation and abnormal differentiation of epidermal keratinocytes with polymorphonuclear leukocytes, activated lymphocytes, and other chronic inflammatory cells within the affected dermis and epidermis.[1] Prevention of aberrant keratinocyte formation was thought to be the key to controlling psoriasis until about 10 years ago when the emphasis shifted to regulation of an underlying immune mechanism. Immune-modulating agents such as cyclosporine,[2,3] tacrolimus,[4] etretinate,[5] and CD4 monoclonal antibodies[6] can produce clinical improvement in psoriasis by focusing attention on the T cell. Likewise, remittive therapies like psoralen plus ultraviolet A can induce T-cell apoptosis of lymphocytes.[7] However, many of these agents have been shown to also have a direct effect on keratinocytes, leaving unresolved the issue of the primary cause of the disease. Denileukin diftitox (ONTAK, DAB_{389}IL-2) has been shown to have antipsoriatic activity, and it reacts with activated lymphocytes, not with keratinocytes.[8]

Denileukin diftitox is an interleukin 2 receptor (IL-2R)–targeted fusion protein produced by recombinant DNA techniques.[9] The denileukin diftitox

From Washington University School of Medicine, St Louis,[a] Brown University School of Medicine, Providence,[b] Hospital of the University of Pennsylvania, Philadelphia,[c] UMDNJ–Robert Wood Johnson Medical School, New Brunswick,[d] Lawrence Clinical Research, Lawrenceville,[e] Psoriasis Treatment Center of Central New Jersey, East Windsor,[f] and Seragen, Inc, San Diego.[g]

Supported by a grant from Seragen, Inc.

Dr Muglia has been a paid consultant to Ligand Pharmaceuticals Inc (the owner of Seragen, Inc). Dr Bacha was an employee of Seragen, Inc, at the time of this study and has served as a paid consultant to Ligand Pharmaceuticals Inc.

Accepted for publication May 21, 2001.

Reprint requests: Ann Martin, MD, Department of Dermatology, Room 113, Washington University School of Medicine, 4570 Children's Place, St Louis, MO 63110.

0190-9622/2001/$35.00 + 0 **16/1/117852**
doi:10.1067/mjd.2001.117852

molecule consists of IL-2 and a portion of the diphtheria toxin molecule. Denileukin diftitox binds to the IL-2 receptor (IL-2R) and is rapidly internalized via receptor-mediated endocytosis. Subsequently, the enzymatic domain of the toxin molecule is released into the cell cytosol resulting in inhibition of protein synthesis and, ultimately, in cell death. Denileukin diftitox is selectively targeted to activated lymphocytes.[10] These cells bear the trimeric form of the IL-2R, which has a 1000-fold greater affinity for IL-2 than does the receptor found on resting cells.

Denileukin diftitox was originally developed clinically for the treatment of IL-2R–expressing malignancies.[11-14] It is currently approved for use in patients with persistent or recurrent cutaneous T-cell lymphoma (CTCL) whose malignant cells express the CD25 component of the IL-2 receptor when used at a dose of 9 or 18 μg/kg per day for 5 consecutive days. At these doses, the overall response in a recent study of patients with CTCL was 30% (21/71), with 10% of patients achieving a complete response and 20% a partial response.[11]

Two clinical trials have been completed with denileukin diftitox in patients with long-standing moderate to severe psoriasis.[15,16] The first study was an open-label cohort dose-escalation trial in 24 patients evaluating an infusion of 2, 4, 6, or 9 μg/kg per day for 5 consecutive days every 4 weeks for up to 6 months. Mean Psoriasis Area and Severity Index (PASI)[17] scores for the study population as a group steadily declined over the study period, and half of the patients had a greater than 50% improvement in their PASI score. Response rates by dose panel suggested a possible dose-response trend. There was a greater than 50% improvement in PASI score in 2 of 6 (33%) of the patients treated with 2 μg/kg per day, 5 of 10 (50%) of those treated with 4 or 6 μg/kg per day, and 5 of 8 (63%) of those treated with 9 μg/kg per day. Reported adverse events were mild to moderate in severity and consisted predominantly of flu-like symptoms (chills/fever, nausea/vomiting, headache, myalgia/arthralgia), dizziness, increased pruritus, and transient transaminase elevations. Five patients (2 receiving 2 μg/kg daily and 3 receiving 9 μg/kg daily) discontinued because of adverse events (rash, back pain, sepsis, arthralgia, hyperthyroidism).

A desire to shorten the time to response by alteration of the dosing regimen led to the initiation of a second trial. This study was a double-blind, placebo-controlled trial with a dosing schedule of 3 consecutive days every week for 4 weeks. In addition, a slightly higher range of doses was examined (5, 10, and 15 μg/kg daily). Thirty-four percent of patients (10/29) who received denileukin diftitox improved by at least 50% at some time during the 8 weeks of the study, and the response rates showed no evidence of a dose-dependence. The response rates for patients treated with 5, 10, and 15 μg/kg per day doses were 36% (4/11), 40% (4/10), and 25% (2/8), respectively. However, this dosing schedule was not well tolerated, and 10 patients (3 at 5 μg/kg daily, 3 at 10 μg/kg daily, and 4 at 15 μg/kg daily) discontinued treatment because of adverse events (4 rash, 2 hypotension, 1 facial edema, 1 fever/chills, 1 nausea, and 1 vasospasm). In addition, one patient at the 5 μg/kg daily dose level in this trial experienced a lower extremity arterial and venous thrombosis. This patient had a medical history significant for heavy smoking and alcohol abuse along with a family history of cardiovascular disease, hypertension, and diabetes, and the relationship of this event to treatment with denileukin diftitox was uncertain. After submission of data to support the ambiguity of the relationship of this event to denileukin diftitox administration and discussion with the Food and Drug Administration, a decision was made to close the protocol, analyze the data, and initiate a new trial.

The study reported herein was conducted to collect additional safety and efficacy data at lower doses (0.5 to 5 μg/kg daily) of denileukin diftitox administered on a schedule (3 consecutive days every other week for 8 weeks) intermediate to those studied previously.

PATIENTS AND METHODS

This was a cohort dose-escalation multicenter study to evaluate the safety, tolerability, pharmacokinetics, and antipsoriatic effects of denileukin diftitox at 3 dose levels in patients with severe plaque-type psoriasis. The study was conducted under an Investigational New Drug Application and was approved by the Institutional Review Board of each site. Written informed consent was obtained from each patient before enrollment.

Study eligibility

To enroll in the study, patients with severe plaque-type psoriasis had to be 18 years of age or older and be willing to give informed consent and comply with protocol requirements. Severe disease was defined as (1) greater than 20% body surface area involved with plaque-type lesions or (2) an extent and location of disease that significantly adversely affected routine activities of daily living. Patients with pustular or erythrodermic psoriasis were excluded. Patients had to be in good general health with hepatic transaminase levels less than 1.5 times the upper limit of normal (ULN), serum albumin of 3.0 g/dL or greater, serum creatinine less than 1.5 times ULN or estimated creatinine clearance greater than 60

mL/min, and prothrombin time or partial thromboplastin time 1.25 times ULN or less. Patients excluded from study were those who had active infections including HIV and hepatitis B or C, a history of clinically significant cardiovascular disease or coagulation disorder, an active malignancy, or previous exposure to denileukin diftitox. Pregnant or lactating women or individuals of childbearing potential unwilling to practice adequate contraception were not eligible. In addition, there was a washout period for antipsoriatic therapies: 8 weeks for cyclosporine or methotrexate; 4 weeks for investigational drugs, PUVA, UVB, coal tar, vitamin D_3, retinoids, or oral or parenteral steroids; and 2 weeks for topical agents such as vitamin D_3, anthralin, corticosteroids, or keratolytic agents.

Treatment plan

The study was conducted at 6 sites and planned to enroll between 10 and 15 patients at each of 3 dose levels (0.5, 1.5, and 5 μg/kg daily) in a cohort dose-escalating manner. The safety for each cohort was to be assessed and considered acceptable before enrollment into the next higher cohort. Doses were administered as an intravenous infusion lasting at least 15 minutes for 3 consecutive days every other week for 8 weeks for a total of 12 doses. Patients were permitted to receive acetaminophen or aspirin and antihistamine before infusion of the study drug.

Safety monitoring

Safety was assessed at the screening visit and on a weekly basis during the 8 weeks of the study by routine laboratory evaluations (hematology, clinical chemistry, and urinalysis), physical examinations, and adverse event reports obtained either by solicitation from patients or by observation of medical staff. Patients were contacted by telephone 3 months after their last dose for a final safety evaluation.

Response evaluation

Antipsoriatic activity was assessed by clinical evaluation of skin changes by designated response assessors. These individuals used the PASI scoring system (72-point total) to record and measure the clinical severity of psoriasis for each patient. The assessor's global impression of the severity of the patient's psoriasis (Physician's Global Assessment or PGA) was recorded by means of a 5-point (0-4) scale (asymptomatic, mild, moderate, severe, or very severe). Assessments were performed at the screening visit and then weekly during the 8 weeks of the study. Patients with a decrease of 30% or more in PASI score from baseline (average of scores obtained at the screening visit and on day 1 before dosing) at the end of the 8-week period were to be followed monthly until the patient required use of antipsoriatic medication, returned to his/her baseline PASI score, or completed 1 year of follow-up, whichever occurred first.

Antibody measurements

Blood samples were drawn for measurement of anti–denileukin diftitox and anti–IL-2 antibody levels before the first, seventh, and tenth doses and during the final observation week. Antibody levels were measured by means of an enzyme-linked immunosorbent assay (ELISA) with plates that were coated with either denileukin diftitox or IL-2 and incubated with 5-fold serial dilutions of serum.[16]

Denileukin diftitox serum levels

Blood samples were drawn at 5, 10, and 15 minutes after the start of the denileukin diftitox infusion, and 5, 15, 30, 60, 90, 120, 180, and 240 minutes after completing the infusion during the first, third, eleventh, and twelfth doses. Serum concentrations of immunoreactive denileukin diftitox were determined in a standard asymmetric sandwich ELISA using 2 antibodies: one to the diphtheria toxin portion and one to the IL-2 portion of the molecule.[16] A processing step that dissociates denileukin diftitox from bound antibodies allows the determination of total circulating levels of denileukin diftitox. The lower limit of quantitation of denileukin diftitox in serum is approximately 0.2 ng/mL.

RESULTS

Key demographic factors and baseline disease characteristics for the study population are summarized in Table I. A total of 35 patients were enrolled into the 3 groups (10 patients at 0.5 μg/kg daily, 10 patients at 1.5 μg/kg daily, and 15 patients at 5 μg/kg daily). The 3 groups were similar in demographic and disease factors. Although group 2 appears to have slightly less severe disease at baseline than groups 1 or 3, the difference is not significant because a single patient with a 4.6 baseline PASI was enrolled in group 2 and is responsible for skewing the group mean. This patient qualified for the trial because of the extent and location of disease and its adverse impact on routine activities of daily living.

Overall, 77% (27/35) of patients enrolled in the study were considered to have severe to very severe disease. The remaining patients qualified for the study because the extent and location of their psoriasis significantly affected their routine activities of daily living. Many patients had either a clinical variant of psoriasis (34%) or psoriatic arthritis (26%) (see Table I). Patients in the study had received a median

Table I. Patient demographics and baseline disease characteristics

Parameter	0.5 μg/kg daily, n = 10	1.5 μg/kg daily, n = 10	5 μg/kg daily, n = 15	All, n = 35
Patients				
Age (y)				
Mean	51	50	49	50
Range	32-80	23-67	37-67	23-80
Sex, No. (%)				
Male	6 (60)	5 (50)	9 (60)	20 (57)
Female	4 (40)	5 (50)	6 (40)	15 (43)
Race, No. (%)				
White	9 (90)	10 (100)	14 (93)	33 (94)
Black	0 (0)	0 (0)	1 (7)	1 (3)
Asian	1 (10)	0 (0)	0 (0)	1 (3)
Disease				
Duration (y)				
Mean	23	16	24	22
Range	7-54	1-43	7-43	1-54
Variants, No. (%)				
None	5 (50)	6 (60)	12 (80)	23 (66)
Seborrheic psoriasis	5 (50)	4 (40)	1 (7)	10 (29)
Guttate psoriasis	5 (50)	3 (30)	2 (13)	10 (29)
Reiter's disease	1 (10)	0 (0)	0 (0)	1 (3)
Psoriatic arthritis, No. (%)				
No	6 (60)	9 (90)	11 (73)	26 (74)
Yes	4 (40)	1 (10)	4 (27)	9 (26)
Baseline PASI score				
Mean	26.0	20.0	24.2	23.5
Range	8.8-56	4.6-32	12.3-43	4.6-56
Baseline PGA, No. (%)				
Mild	0 (0)	1 (10)	0 (0)	1 (3)
Moderate	1 (10)	3 (30)	3 (20)	7 (20)
Severe	7 (70)	6 (60)	11 (73)	24 (69)
Very severe	2 (20)	0 (0)	1 (7)	3 (9)

Table II. Patient disposition

	0.5 μg/kg daily, n = 10, No. (%)	1.5 μg/kg daily, n = 10, No. (%)	5 μg/kg daily, n = 15, No. (%)	All, n = 35, No. (%)
Completed study	9 (90)	8 (80)	12 (80)	29 (83)
Withdrew				
Adverse events	1 (10)	1 (10)	3 (20)	5 (14)
Lack of improvement	0 (0)	1 (10)	0 (0)	1 (3)

of 5 previous therapies (range, 2-10). All had used topical therapy such as corticosteroids, vitamin D, or anthralin; 89% received UV light treatment with or without psoralen or other agents; and 57% had used cyclosporine, methotrexate, or etretinate.

Twenty-nine (83%) of the 35 patients enrolled in the study completed the 8-week study (Table II). One patient discontinued for lack of improvement and 5 patients withdrew for adverse events. All 35 patients are included in the intent-to-treat analysis.

Response evaluation

The primary parameter for measuring antipsoriatic effects in this study was the change in PASI score from baseline previously defined as the average of the screening and day 1 scores. As shown in Fig 1, there was a mean decrease in PASI scores for each of the 3 dose groups. However, only patients in the 5 μg/kg daily dose group showed consistent improvement with time, leading to a mean decrease of 35% at the last study visit (day 50) approximately 1 week after the last dose.

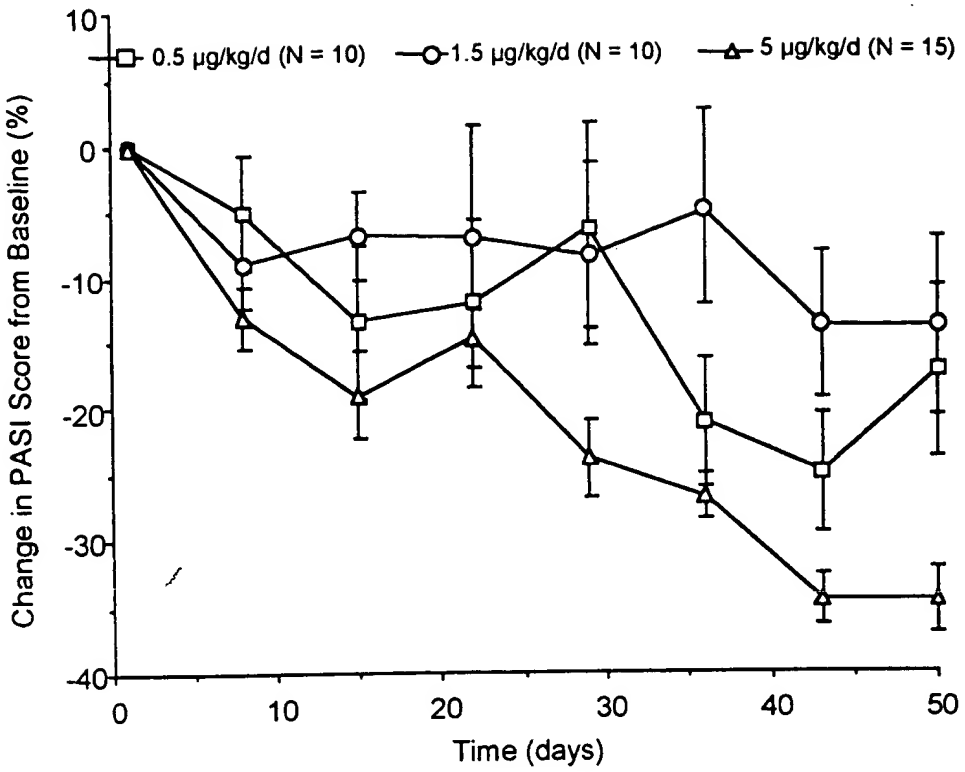


Fig 1. Change in PASI scores over study period for 3 dose groups (mean ± standard error of mean). Denileukin diftitox was administered on days 1 to 3, 15 to 17, 29 to 31, and 43 to 45. *N* equals total number of patients for whom data were available. Number of patients with data at each time point varied.

Table III. Patients with improvement in PASI or PGA

	0.5 µg/kg daily, n = 10, No. (%)	1.5 µg/kg daily, n = 10, No. (%)	5 µg/kg daily, n = 15, No. (%)	All, n = 35, No. (%)
PASI (≥50% decrease)	0 (0)	1 (10)	7 (47)	8 (23)
PGA (≥1 grade decrease)	5 (50)	3 (30)	10 (67)	18 (51)
PGA (≥2 grade decrease)	0 (0)	0 (0)	4 (27)	4 (11)

Overall, 21 (60%) of the 35 patients achieved a 30% decrease or greater in PASI score at some point during the study, whereas 8 patients (23%) met the criterion of a 50% or greater reduction. Seven (47%) of the 15 patients in the 5 µg/kg daily group had a 50% or greater reduction in PASI score (Table III). It should be noted, however, that one of these patients (patient 602) began to show improvement in disease symptoms, but then experienced a rash requiring oral corticosteroids and discontinuation from the study. The eighth responder (patient 411) was in the 1.5 µg/kg daily group. This individual had a low baseline PASI score of 4.6. The PASI scores of one patient in the 1.5 µg/kg daily dose group (patient 307) worsened in 4 of 6 study visits, but the patient remained in the study.

PASI scores over time for all 8 responders are illustrated in Fig 2. Seven patients still showed improvement at their last assessment, with 3 patients (606, 607, and 613) followed up for more than a month beyond their last dose. Additional data beyond the time points shown in Fig 2 are not available. Interestingly, patient 613 did not achieve a decrease of 50% or greater in PASI score until 33 days after his last dose.

As shown in Table III, 51% (18/35) of patients had a decrease from baseline in PGA by at least one grade at some time during the study, with a comparable number of patients meeting this criteria across the 3 dose groups, but the 4 patients who had a two-grade decrease were all in the 5 µg/kg daily group.

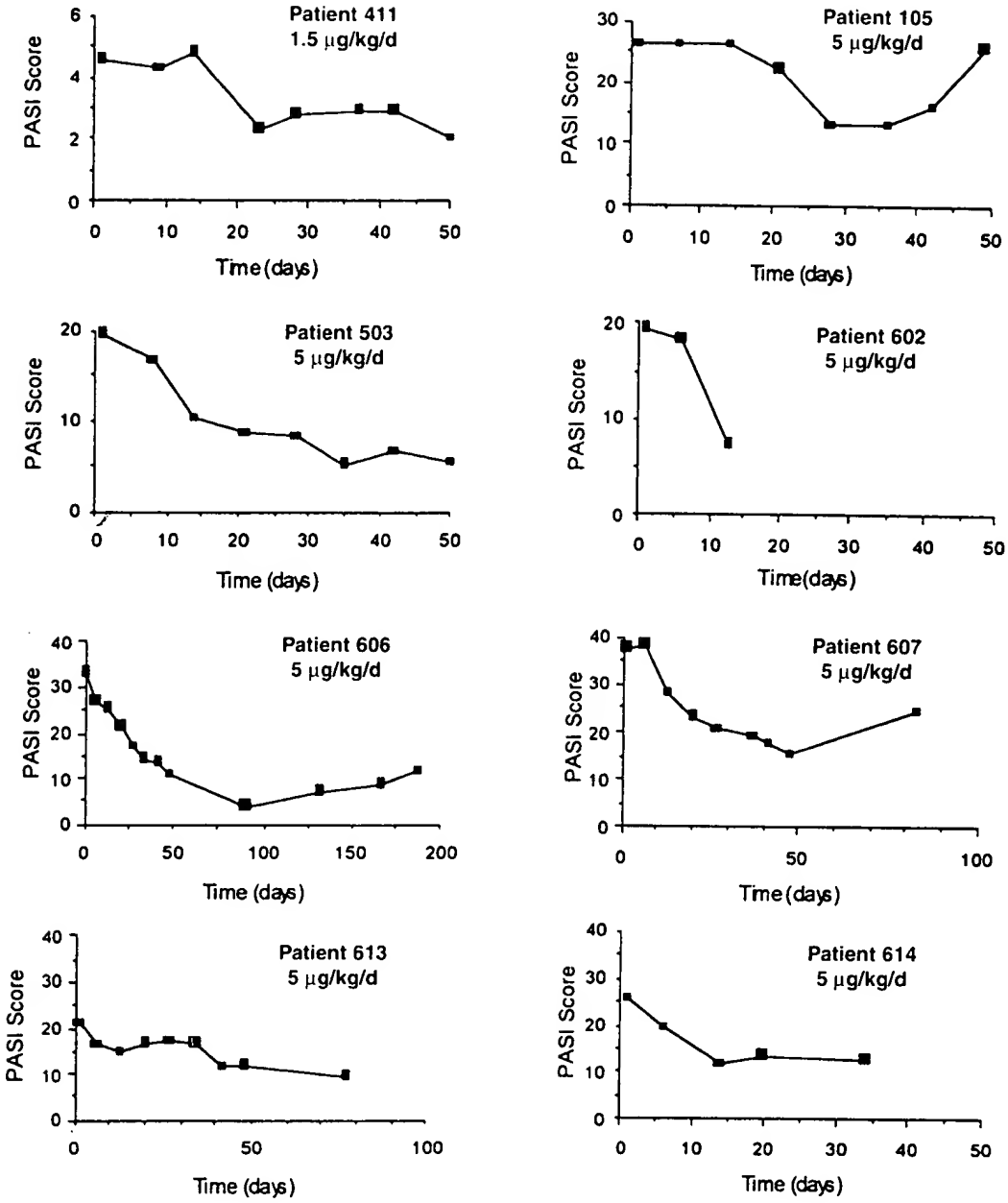


Fig 2. PASI scores over time for the 8 patients who achieved ≥ 50% decrease in PASI score from baseline. Day 50, approximately 1 week after last dose, was considered final study visit. Patients 602 and 614 discontinued because of adverse events after 3 and 6 doses, respectively. Final data shown for these patients are 10 and 18 days, respectively, after last dose. Patients 606, 607, and 613 were followed up beyond last dose for an additional 145, 39, and 33 days, respectively.

Safety evaluation

Five (14%) of the 35 patients discontinued for adverse events, one in each of the lower dose groups and 3 of 15 in the highest dose group. One patient (0.5 µg/kg daily) discontinued for chest pain and was later diagnosed with bronchitis. One patient (1.5 µg/kg daily) with a history of uncontrolled diabetes experienced cellulitis and left the study after a brief hospitalization. The events experienced by both of these patients were considered to be unrelated to denileukin diftitox by their physicians. Possibly related events in patients in the 5 µg/kg daily dose group included facial edema that cleared within a few hours of the start of diphenhydramine treatment and rash in 2 patients (5 µg/kg daily). A biopsy of the first case of rash showed a perivascular lymphocyte infiltrate of the superficial dermis consistent with a drug eruption. This rash, which evolved from gener-

Table IV. Mild (grade 1 and 2) adverse events experienced by 2 or more patients considered related to treatment

Adverse event	0.5 μg/kg daily, n = 10, No. (%)	1.5 μg/kg daily, n = 10, No. (%)	5 μg/kg daily, n = 15, No. (%)	All, n = 35, No. (%)
Fever/chills	1 (10)	1 (10)	6 (40)	8 (23)
Asthenia	2 (20)	0 (0)	5 (33)	7 (20)
Pain	1 (10)	2 (20)	4 (27)	7 (20)
Rash	1 (10)	0 (0)	4 (27)	5 (14)
Nausea/vomiting	2 (20)	0 (0)	2 (13)	4 (11)
Headache	0 (0)	2 (20)	1 (7)	3 (9)
Pruritus	0 (0)	0 (0)	2 (13)	2 (6)
Insomnia	0 (0)	0 (0)	2 (13)	2 (6)
Myalgia	0 (0)	0 (0)	3 (20)	3 (9)
Psoriasis	1 (10)	1 (10)	1 (7)	3 (9)
Edema	0 (0)	0 (0)	3 (20)	3 (9)
Transaminase increase	0 (0)	0 (0)	3 (20)	3 (9)
LDH increase	0 (0)	0 (0)	2 (13)	2 (6)
Myasthenia	0 (0)	0 (0)	2 (13)	2 (6)
Weight increase	0 (0)	0 (0)	2 (13)	2 (6)

LDH, Lactate dehydrogenase. The following events considered to be related to treatment were experienced by 1 patient each: allergic reaction, anorexia, arthralgia, capillary fragility increased, chest pain, creatinine clearance decrease, diarrhea, dizziness, eosinophilia, hypotension, infection (sinusitis), injection site reaction, lab test abnormality, leukocytosis, leukopenia, lymphadenopathy, nervousness (anxiety), skin discoloration, sweating, and taste perversion.

alized erythroderma and cutaneous edema into an exfoliative dermatitis, required treatment with oral corticosteroids. The biopsy specimen from the second rash revealed features consistent with psoriasis but did not exclude the diagnosis of drug reaction. This individual's skin eruption and eosinophilia cleared without specific therapy.

The most common adverse events experienced by patients in this trial (see Table IV) were fever/chills (23%; with no temperatures above 38°C), asthenia (20%), pain (20%; primarily back pain with infusion), rash (14%; included generalized rash, macular papular rash, and vesicular rash), and nausea/vomiting (11%). Constitutional events such as asthenia and fever/chills, events related to skin reactions such as generalized pruritus and rash, and increased transaminase levels were experienced more frequently by patients in the 5 μg/kg daily group than in the other two lower dose groups. Constitutional symptoms usually began on a dosing day and lasted 1 to 4 days.

Acute events associated with infusion of denileukin diftitox in this study were usually limited to back pain. Investigators considered the one case of facial edema and two cases of rash to be manifestations of delayed hypersensitivity-type reactions. A case of generalized edema in the 5 μg/kg daily dose group was considered to be probably related to study drug. There was one report of a mild vascular leak syndrome (VLS) in a patient in the 5 μg/kg daily dose group that occurred at day 7 and was also believed to be treatment related. VLS is defined as the simultaneous occurrence of at least 2 of the following: edema, hypoalbuminemia (≤ 2.8 g/dL), or hypotension occurring between days 1 and 14 of the beginning of a treatment cycle. The patient with VLS in this study had a slight reduction in albumin (nadir of 3.1 g/dL), ankle edema, and a 6% weight gain, which did not require treatment.

Table V. Number of patients with moderate (grade 3) and severe (grade 4) adverse events (n = 35)

	Related events (No.)		Unrelated events (No.)	
Adverse event	Moderate	Severe	Moderate	Severe
Cellulitis	0	0	0	1
Flu-like syndrome	0	0	1	0
Kidney calculus	0	0	1	0
Leukopenia	0	1	0	0
Pruritus	0	0	1	0
Transaminase elevation	1	0	0	0

All of the adverse experiences and changes in laboratory parameters reported in this study were mild (grade 1 or 2) with the exception of those events described below. Four patients experienced moderately severe (grade 3) events (Table V; one patient in the 0.5 μg/kg daily group with a kidney calculus, two

Table VI. Antibody levels with time

Titer	Day 1			Day 50		
	0.5 μg·kg daily, n = 10, No. (%)	1.5 μg/kg daily, n = 10, No. (%)	5 μg/kg daily, n = 14, No. (%)	0.5 μg/kg daily, n = 10, No. (%)	1.5 μg/kg daily, n = 7, No. (%)	5 μg/kg daily, n = 10, No. (%)
Anti–denileukin diftitox antibodies						
<1:5	2 (20)	6 (60)	2 (14)	2 (20)	0 (0)	0 (0)
1:5	5 (50)	2 (20)	8 (57)	0 (0)	0 (0)	0 (0)
1:25	3 (30)	2 (20)	3 (21)	0 (0)	0 (0)	0 (0)
1:125	0 (0)	0 (0)	1 (7)	3 (30)	0 (0)	0 (0)
1:625	0 (0)	0 (0)	0 (0)	1 (10)	5 (71)	4 (40)
1:3125	0 (0)	0 (0)	0 (0)	4 (40)	2 (29)	5 (50)
1:15,625	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)
>1:15,625	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Anti–IL-2 antibodies						
<1:5	8 (80)	9 (90)	11 (79)	5 (50)	1 (14)	1 (10)
1:5	2 (2)	1 (10)	3 (21)	4 (40)	3 (43)	3 (30)
1:25	0 (0)	0 (0)	0 (0)	0 (0)	1 (14)	4 (40)
1:125	0 (0)	0 (0)	0 (0)	1 (10)	2 (29)	2 (20)
1:625	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
1:3125	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
1:15,625	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
>1:15,625	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

patients in the 1.5 μg/kg daily group [one with pruritus and one with flu-like syndrome], and one patient in the 5 μg/kg daily group with a transient increase in transaminase levels [ALT = 176 U/dL]). In addition, two severe (grade 4) events were reported: the case of cellulitis in the diabetic patient described above and a transient case of leukopenia in a patient in the lowest dose group whose lymphocyte count dropped after an upper respiratory tract infection from 1110 cells/μL on day 23 to 390 cells/μL before dosing on day 29. The count subsequently returned to 930 cells/μL on day 38 and remained stable through the next dosing cycle. The only AE-related hospitalization was for the patient with cellulitis.

Immunogenicity

Antibody titers were measured at timepoints before and during the study. At baseline, 26% of the patients (9/34) whose samples were available had anti–denileukin diftitox antibody titers of 1:25 or 1:125 (Table VI). This is believed to be because of previous diphtheria toxoid immunization. Anti-diphtheria toxin antibodies have been previously shown to cross-react with denileukin diftitox. An increase in anti–denileukin diftitox antibody titers to 1:625 or more was first seen after 6 doses (day 29). These titers usually did not change with subsequent dosing and, by the end of the study (day 50), all of the patients in the 1.5 and 5 μg/kg daily groups had titers of at least 1:625. In contrast, only half of the patients in the 0.5 μg/kg daily group had titers as high as 1:625, with two patients not developing any antibodies to denileukin diftitox. These results are similar to those of a previous phase III CTCL study in which specific neutralizing antibodies and anti–denileukin diftitox ELISA titers were found to increase in parallel after exposure to the study drug.[11]

Although all of the patients in this study had low or undetectable (≤ 1:5) anti–IL-2 titers at baseline, 43% of patients in the 1.5 μg/kg daily group and 60% of those in the 5 μg/kg daily group developed titers of 1:25 or 1:125 by day 50 (Table VI). In contrast, only one (10%) of the patients in the 0.5 μg/kg daily group developed antibodies to IL-2 (titer 1:125).

Denileukin diftitox serum levels

Serum denileukin diftitox concentrations were measured during the first and last week of dosing. As shown in Fig 3, serum concentrations after the first administration were dose-proportional, with mean peak concentrations ranging from 6.9 ng/mL (0.5 μg/kg daily) to 66.5 ng/mL (5 μg/kg daily). The mean peak at the 0.5 μg/kg daily dose was below the target level of more than 10 ng/mL predicted to be necessary for bioactivity based on in vitro cell culture data.[9]

As shown in Fig 4, no accumulation was observed between the first and third doses of the same dosing week (day 1 vs day 3, and day 43 vs day 45) even in

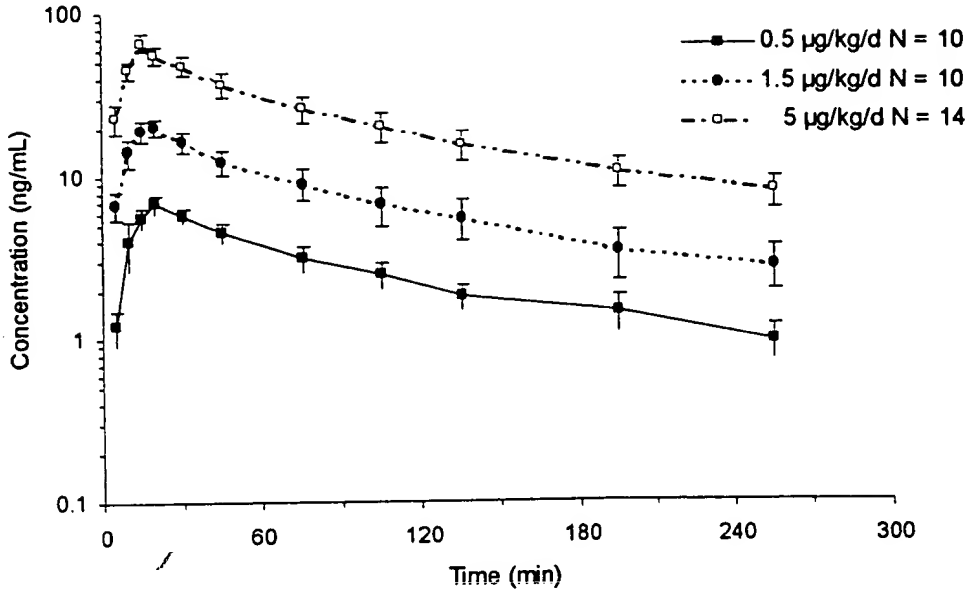


Fig 3. Serum concentrations of denileukin diftitox after first dose (day 1) for the 3 dose groups (mean ± standard error of mean). *N* equals total number of patients for whom data were available. Number of patients with data at each time point varied.

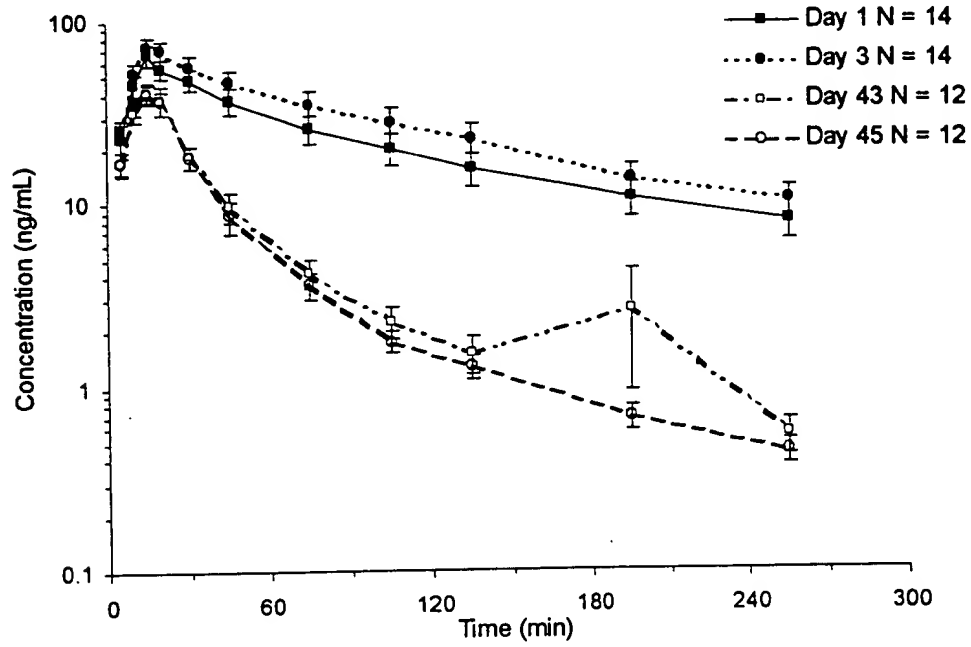


Fig 4. Serum concentrations of denileukin diftitox after first (day 1), third (day 3), eleventh (day 43), and twelfth (day 45) doses for the 5 µg/kg daily group (mean ± standard error of mean). *N* equals total number of patients for whom data were available. Number of patients with data at each time point varied.

the highest dose group (5 µg/kg daily). As also shown in Fig 4, development of antibodies correlated with a significant decrease in serum concentrations between the initial dose in the first and last dosing weeks (day 1 vs day 43). Mean peak levels for the 5 µg/kg daily group decreased from 66.5 ng/mL on day 1 to 41.7 ng/mL on day 43 with a comparable decrease in overall exposure.

Table VII. Peak serum levels and antibody titers for patients with a ≥50% decrease in PASI score from baseline

	Day 1			Day 43		
Patient	Peak levels (ng/mL)	Anti–denileukin diftitox titer	Anti–IL-2 titer	Peak levels (ng/mL)	Anti–denileukin diftitox titer	Anti–IL-2 titer
1.5 μg/kg daily						
411	>25.6	<1:5	<1:5	13.9	1:625	1:125
5 μg/kg daily						
105	58.1	1:25	1:5	40.0	1:3125	1:25
503	59.9	1:5	1:5	33.2	>1:15,625	1:125
602	83.6	<1:5	<1:5	N/A	N/A	N/A
606	55.0	1:25	<1:5	33.4	1:3125	<1:5
607	>51.2	1:5	<1:5	95.7	1:625	1:25
613	62.0	1:25	1:5	68.9	1:625	1:25
614	57.9	1:5	<1:5	N/A	N/A	N/A

N/A, Not available.

These lower levels did not, however, drop below the target level (>10 ng/mL) noted above. It is also important to note that although the development of antibodies generally corresponded with decreased denileukin diftitox serum levels, the presence of these antibodies did not preclude response. Of the 8 individuals with a greater than 50% decrease in PASI score, 3 had baseline anti–denileukin diftitox antibody titers of 1:25 (Table VII). In addition, all 6 patients for whom data are available had anti–denileukin diftitox antibody titers of 1:625 to >1:15,625 by the end of dosing (day 43), and 5 of the 6 also showed an anti–IL-2 response by that time. There was no apparent correlation between response and antibody levels.

DISCUSSION

Although denileukin diftitox has received accelerated approval in the United States for the treatment of CTCL, the clinical development of this agent for the treatment of autoimmune disease is in an early stage. However, denileukin diftitox has now been shown to have antipsoriatic activity in patients with moderate to severe psoriasis in 3 trials using different regimens. In the two previously reported trials, dose response for improvement in psoriatic symptoms could not be demonstrated using the regimens examined.[8,15,16] In contrast, this trial established a dose and schedule that is active. No patients (0/10) in the 0.5 μg/kg daily group and only 1 patient (1/10) in the 1.5 μg/kg daily group had a 50% or greater decrease in PASI score from baseline. However, 7 of the 15 patients in the 5 μg/kg daily group met this criterion.

The safety profile reported in this study is similar to the profiles in the earlier two studies. The most frequently reported events are a group of flu-like or constitutional symptoms (asthenia, fever/chills, nausea, and headache). However, the frequency or severity of many adverse events reported in this trial at the 5 μg/kg daily dose level for 3 consecutive days every other week were lower than those reported in the earlier trial[16] using a more intensive dosing schedule (3 consecutive days every week). For example, the frequency of fever or chills was 40% for this trial with all events being grade 1 versus 64% for the previous trial with half of the events being grade 2. A similar relationship was observed with respect to the frequency and severity of asthenia (33% primarily grade 1 versus 45% primarily grade 2, respectively). One case of VLS occurred at the 5 μg/kg daily dose. This VLS incidence in the high-dose panel of 0.7% (1/15) is lower than that of a previous study of patients with CTCL, in which VLS occurred at a rate of 25% in patients treated at doses of 9 or 18 μg/kg daily.[11]

In conclusion, this study has identified a minimum dose for antipsoriatic activity of denileukin diftitox at 5 μg/kg daily using this schedule. The response rate at this dose level was comparable to that reported for other studies, and this dosing regimen was better tolerated. Further optimization of the dosing schedule may be desirable, however, because a less frequent dosing schedule would be more acceptable to both patients and physicians. In addition, periodic dosing to maintain response is an area for future study.

REFERENCES

1. Weinstein GD, Krueger JG. An overview of psoriasis. In: Weinstein GD, Gottlieb AB, editors. Therapy of moderate-to-severe psoriasis. Portland (OR): National Psoriasis Foundation; 1993. p. 1-22.
2. Baker BS, Griffiths CEM, Lambert S, Powles AV, Leonard JN,

Valdimarsson H, et al. The effects of cyclosporin A on T lymphocytes and dendritic cell sub-populations in psoriasis. Br J Dermatol 1987;116:503-10.
3. Bos JD, Meinardi MM, vanJoost T, Henle F, Powles AV, Fry L. Use of cyclosporin in psoriasis. Lancet 1989;2:1500-2.
4. The European FK506 Multicentre Psoriasis Study Group. Systemic tacrolimus (FK506) is effective for the treatment of psoriasis in a double-blind, placebo-controlled study. Arch Dermatol 1996;132:419-23.
5. Gottlieb SL, Hayes E, Gilleaudeau P, Cardinale I, Gottlieb AB, Krueger JG. Cellular actions of etretinate in psoriasis: enhanced epidermal differentiation and reduced cell-mediated inflammation are unexpected outcomes. J Cutan Pathol 1996;23:404-18.
6. Prinz J, Braun-Falco O, Meurer M, Daddona P, Reiter C, Reiber P, et al. Chimaeric CD4 monoclonal antibody in treatment of generalized pustular psoriasis. Lancet 1991;338:320-1.
7. Vallat VP, Gilleaudeau P, Battat L, Wolfe J, Nabeya R, Heftler N, et al. PUVA bath therapy strongly suppresses immunological and epidermal activation in psoriasis: a possible cellular basis for remittive therapy. J Exp Med 1994;180:283-96.
8. Gottlieb SL, Gilleaudeau P, Johnson R, Estes L, Woodworth TG, Gottlieb AB, et al. Response of psoriasis to a lymphocyte-selective toxin (DAB_{389}IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. Nat Med 1995;1:442-7.
9. Williams DP, Snider CE, Strom TB, Murphy JR. Structure/function analysis of interleukin-2-toxin (DAB_{486}IL-2): fragment B sequences required for the delivery of fragment A to the cytosol of target cells. J Biol Chem 1990;265:11885-9.
10. Waters CA, Schimke PA, Snider CE, Itoh K, Smith KA, Nichols JC, et al. Interleukin 2 receptor-targeted cytotoxicity. Receptor binding requirements for entry of a diphtheria toxin-related interleukin 2 fusion protein into cells. Eur J Immunol 1990;20:785-91.
11. Olsen E, Duvic M, Frankel A, Kim Y, Martin A, Vonderheid E, et al. Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T cell lymphoma. J Clin Oncol 2001;19:376-88.
12. Saleh MN, LeMaistre CF, Kuzel TM, Foss F, Platanias LC, Schwartz G, et al. Antitumor activity of DAB_{389}IL-2 fusion toxin in mycosis fungoides. J Am Acad Dermatol 1998;39:63-73.
13. LeMaistre CF, Saleh MN, Kuzel TM, Foss F, Platanias LC, Schwartz G, et al. Phase I trial of a ligand fusion-protein (DAB_{389}IL-2) in lymphomas expressing the receptor for interleukin-2. Blood 1998;91:399-405.
14. Duvic M, Cather J, Maize J, Frankel AE. DAB_{389}IL2 diphtheria fusion toxin produces clinical responses in tumor stage cutaneous T cell lymphoma. Am J Hematol 1998;58:87-90.
15. Gottlieb AB, Bacha P, Parker K, Strand V. Use of interleukin-2 fusion protein, DAB_{389}IL-2, for the treatment of psoriasis. Dermatol Ther 1998;5:48-63.
16. Bagel J, Garland WT, Breneman D, Holick M, Littlejohn TW, Crosby D, et al. Administration of DAB_{389}IL-2 to patients with recalcitrant psoriasis: a double-blind, phase II multicenter trial. J Am Acad Dermatol 1998;38:938-44.
17. Fredriksson T, Pettersson U. Severe psoriasis: oral therapy with a new retinoid. Dermatologica 1978;157:238-44.

Patricia Bacha, Seren E. Forte, Stuart J. Perper, David E. Trentham[Δ] and Jean C. Nichols

Seragen, Inc., Hopkinton and Division of Rheumatology[Δ], Beth Israel Hospital, Boston

Anti-arthritic effects demonstrated by an interleukin-2 receptor-targeted cytotoxin ($DAB_{486}IL$-2) in rat adjuvant arthritis

$DAB_{486}IL$-2 is an interleukin-2 receptor-specific cytotoxin which selectively targets and kills cells which bear the high-affinity form of the IL-2 receptor. Since elimination of activated T lymphocytes may be useful in the treatment of rheumatoid arthritis, the effect of $DAB_{486}IL$-2 treatment in an animal model of arthritis was investigated. We demonstrated that rats treated with $DAB_{486}IL$-2 during the induction phase of disease have delayed onset of symptoms and significantly reduced severity of inflammation as well as a depressed proliferative response to mycobacterial stimulation *in vitro*. In addition, the presence of preexisting antibodies to the molecule had no impact on the anti-arthritic effects observed in this model. These data suggest that $DAB_{486}IL$-2 may have therapeutic potential in the treatment of rheumatoid arthritis.

1 Introduction

Two pivotal events of T cell activation include production of IL-2 and appearance of the high-affinity IL-2 receptor on the cell surface [1]. Expression of the high-affinity form of this receptor is restricted to activated T lymphocytes, B lymphocytes and monocytes [2–4]. As a result of its limited distribution, the IL-2 receptor is a target for therapy of those diseases in which activated lymphocytes may play a pathogenic role. In these instances, elimination of IL-2 receptor-positive activated cells should result in selective immunosuppression, while resting, memory and other nonactivated cells should be spared.

Adjuvant arthritis is an autoimmune disease that can be experimentally induced in genetically susceptible rat strains by immunization with mycobacterial adjuvant [5]. The disease is characterized by subacute polyarthritis involving the distal extremities which is similar clinically and pathologically to human rheumatoid arthritis. Similarities include synovitis, pannus formation, cartilage destruction and bone erosion [6]. Involvement of activated T lymphocytes in the pathogenesis of disease has been documented by the observation that adjuvant arthritis, like certain other experimental autoimmune animal models (*e.g.* experimental allergic encephalomyelitis, streptococcal cell wall-induced arthritis, collagen type-II-induced arthritis and non-obese diabetes), can be passively transferred to recipient animals by injection of freshly isolated lymphocytes or selected T cell clones from diseased animals [7].

$DAB_{486}IL$-2 is the product of a fusion gene in which the DNA sequences encoding the receptor-binding domain of native diphtheria toxin have been removed and replaced with DNA sequences encoding human IL-2. The resulting fusion protein contains the enzymatically active domain and membrane associating regions of diphtheria toxin fused to IL-2. This fusion toxin has been shown to be selectively cytotoxic for high-affinity IL-2 receptor-bearing human and murine tumor cells, as well as recently activated PHA-stimulated human peripheral blood mononuclear cells [8–10]. $DAB_{486}IL$-2 represents a new class of biological response modifiers that may play an important role in the management of autoimmune disease in which activation of T lymphocytes plays a pivotal role. This report describes the anti-arthritic activity of $DAB_{486}IL$-2 in the rat adjuvant arthritis model.

2 Materials and methods

2.1 Reagents

$DAB_{486}IL$-2 was expressed in *E. coli* and purified from cellular extracts via immunoaffinity chromatography and high performance liquid chromatography essentially as previously described [11]. Tris-buffered saline consisting of 0.02 M Tris (pH 8.2), 0.15 M NaCl was used as the buffer control. Recombinant human IL-2 was kindly provided by Dr. John R. Murphy (Boston University Medical Center, Boston, MA).

2.2 Animals

Female Lewis rats (100 to 125 g) were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN) and housed in filter-top cages. All animals were maintained in accordance with the guidelines of the Seragen Inc. Institutional Animal Care and Use Committee and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the National Research Council.

2.3 Induction of adjuvant arthritis

Adjuvant arthritis was induced by injecting animals with a 10 mg/ml suspension of killed, dried *Mycobacterium butyricum* (Difco, Detroit, MI) in heavy mineral oil (Sigma Chemical Co., St. Louis, MO). One hundred microliters of

[I 10279]

Correspondence: Patricia Bacha, Seragen, Inc. 97 South St. Hopkinton, MA 01748, USA

0014-2980/92/0707-1673$3.50+.25/0

the suspension was injected intradermally at four to six sites on the lower back while animals were under light methoxyflurane anesthesia.

2.4 Experimental design

Animals were randomly assigned to experimental groups (10 animals/group). Adjuvant injections occurred on day 0. Rats were treated with $DAB_{486}IL$-2 during the induction phase of the disease (days − 1 to 9) or after clinical symptoms of arthritis had developed (days 11 to 21). All animals received a single, daily subcutaneous injection over the back on the days specified. Animals were graded daily for clinical signs of disease. In some studies animals were monitored clinically for up to 3 months, while in other studies animals were euthanized with pentobarbital at the peak of clinical symptoms (day 22) for radiographic and histopathologic evaluation. The right hind limbs from rats in each group were frozen for radiography and the left hind limbs were fixed, sectioned, stained and examined microscopically.

2.5 Scoring of adjuvant arthritis

Each rat was evaluated daily for clinical signs of arthritis. Severity of arthritis was quantified by scoring each paw on a scale of 0 to 4 which indicated the severity of peripheral joint swelling and erythema (0 = no signs of disease, 1 = disease evident in a small number of distal joints of a paw, 2 = disease evident in all the distal joints of the paw, 3 = disease evident in the entire paw and 4 = severe disease evident in the entire paw, [12]). The arthritic index was defined as the sum of the scores of all four paws from each animal with a maximal possible score of 16. Animals were scored by several different blinded observers over the duration of each experiment.

2.6 Radiographic evaluation

At the end of a study, the right hind limbs from randomly chosen rats were amputated above the knee joint and mounted on cardboard using adhesive tape. Limbs were frozen at −20 °C and later imaged on high speed X-ray film at Beth Israel Hospital (Boston, MA). A blinded assessment of the severity of arthritis in each paw was made based on soft tissue swelling and bony proliferation (0 = no evidence of disease, 1 = soft tissue swelling, 2 = soft tissue swelling accompanied by mild new bone formation and 3 = severe soft tissue swelling with extensive new bone formation).

2.7 Histopathology evaluation

The left hind limbs of animals used in the radiographic studies described above were amputated and preserved in Telly's fixative (glacial acetic acid, formaldehyde, and 70 % enthanol in a ratio of 1:2:10). These limbs were sent to Tufts University Veterinary Diagnostic Laboratory (Boston, MA) where they were decalcified, paraffin embedded, sectioned along the midline through the metatarsal region and stained with hematoxylin and eosin. A blinded assessment of the sections was made based upon inflammatory mononuclear cell infiltrate, joint space narrowing and periosteal new bone formation (0 = no evidence of disease, 1 = mild lymphocytic infiltrate, 2 = widespread inflammatory infiltrate with some proliferative osteomyelitis and thickening of the synovial lining and 3 = severe bone destruction and new bone formation and destruction of the synovial lining).

2.8 Assessment of proliferative responses

On day 0 rats (five animals/group) were injected with mycobacterial adjuvant or oil alone and subsequently treated with buffer or $DAB_{486}IL$-2 (0.5 mg/kg) on days 0 to 9. On day 10 or 20 the animals were killed and popliteal lymph nodes were removed under aseptic conditions. The nodes were teased to obtain single-cell suspensions. The cells were cultured at 1×10^6/ml in 96-well U-bottom microtiter plates in RPMI 1640 containing 5 % fetal calf serum and 2×10^{-5} M 2-mercaptoethanol. One hundred microliters of Con A (2 μg/ml), *M. butyricum* (80 μg/ml) or medium alone was then added to quadruplicate wells containing 100 μl of the cell suspension. The cells were incubated for 72 h, pulsed overnight with 0.625 μCi/well [methyl-^{3}H] thymidine (specific activity of 20 Ci/mmol), harvested and assessed for radioactive incorporation.

2.9 Diphtheria toxoid immunization

In a separate study, prior to the induction of adjuvant arthritis, animals were immunized with diphtheria toxoid in order to induce antibodies which might cross-react with the diphtheria toxin portion of the $DAB_{486}IL$-2 molecule. Rats (75 to 100 g) received 100 μg of diphtheria toxoid (Massachusetts State Laboratories, Boston, MA) intramuscularly on 5 consecutive days. Ten days later, blood samples were obtained from each animal and analyzed for anti-$DAB_{486}IL$-2 antibody levels.

2.10 Anti-$DAB_{486}IL$-2 antibody ELISA

Anti-$DAB_{486}IL$-2 antibodies in rat sera were measured by ELISA. Ninety-six-well flexible plates were coated overnight with 1 μg/well of $DAB_{486}IL$-2, blocked with 0.1 % gelatin prepared in 0.05 % Tween-20 (Sigma) in PBS and washed with 0.05 % Tween-PBS. Eight serial fivefold dilutions of each serum sample were added to the coated plate (100 μl/well). The plates were incubated for 1 h at room temperature, washed, incubated with an appropriate dilution of alkaline phosphatase-conjugated affinity-purified goat anti-rat immunoglobulin (Cappel, West Chester, PA), and developed with alkaline phosphatase substrate (Kirkegaard and Perry, Gaithersburg, MD). The antibody titer was determined as the greatest dilution of serum which produced an absorbance at 405 nm of ≥0.1 when analyzed with an automated plate reader.

2.11 Anti-$DAB_{486}IL$-2 neutralizing antibody assay

Antibodies that are capable of neutralizing the biological activity of $DAB_{486}IL$-2 for a human IL-2 receptor-express-

ing cell line were measured in an *in vitro* assay. Twofold dilutions of each serum sample were prepared in RPMI 1640 medium with 15% fetal calf serum and incubated with an equal volume of $DAB_{486}IL$-2 (340 ng/ml) for 1 h at 37 °C. The preincubated mixture was then added to duplicate V-bottom microtiter wells containing 10^5C91/PL cells [13]. The cells were incubated overnight, pulsed for 2 h with 2.5 μCi/ml [^{14}C]leucine (specific activity of 300 mCi/mmol) in leucine-free MEM (Gibco, Grand Island, NY), harvested and assessed for radioactive incorporation. The end point for this assay is defined as the greatest dilution of serum which protects the cells such that their level of incorporation is ≥ 20% of the value for control cells. Results from this assay are reported in units of neutralizing activity. One international neutralizing unit of diphtheria antitoxin is defined as the amount of antibody that will neutralize 2.5 μg of toxin. We have extended this definition to the amount of antibody that will neutralize an equivalent amount on a molar basis (2.7 μg) of DAB_{486}-IL-2.

3 Results

3.1 Clinical course

Rats immunized with mycobacterial adjuvant develop signs of peripheral disease approximately day 10 postimmunization. The severity of swelling and erythema of the paws rapidly increases until day 20 to 25, with individual arthritic indices as high as 10 to 14. Clinical symptoms then gradually decreased to a level which is approximately 50% of the peak by day 40. Daily subcutaneous administration of 0.5 mg/kg of $DAB_{486}IL$-2 from the day prior to adjuvant administration to day 9 postimmunization markedly decreased the severity of inflammation associated with disease. Onset of disease was delayed by approximately 2 days and the degree of peripheral joint swelling and erythema was two- to fourfold less severe than in buffer-treated animals. In the study depicted in Fig. 1, animals treated with $DAB_{486}IL$-2 had a mean arthritic index of 2.5 by day 20, which later decreased so that animals attained a mean score of approximately 0.5 by day 40. In contrast, buffer-treated control animals had a peak mean arthritic index of approximately 9.5 which decreased to only 3 to 4. Subsequent studies have demonstrated that treatment with $DAB_{486}IL$-2 on days 0 to 9 give comparable results to treatment on days −1 to 9. In addition, treatment with either a non-relevant protein, human serum albumin (Alpha Therapeutics, Los Angeles, CA), or IL-2 itself had no impact on clinical symptoms (Fig. 2).

3.2 Radiographic features

Radiographic analysis of the hind limb of buffer-treated control animals, terminated on day 22 postimmunization,

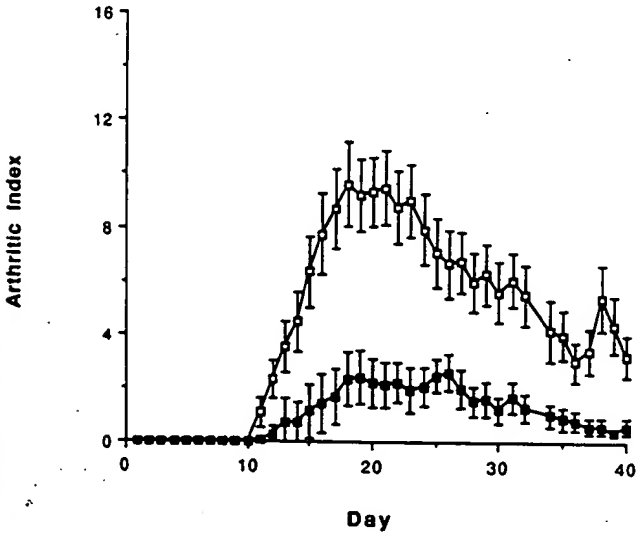


Figure 1. Impact of treatment with $DAB_{486}IL$-2 on induction of adjuvant arthritis. Animals were immunized with adjuvant on day 0 and treated with either Tris-buffered saline (□) or 0.5 mg/kg $DAB_{486}IL$-2 (■) on days −1 to 9. Values are mean ± SEM for 10 animals.

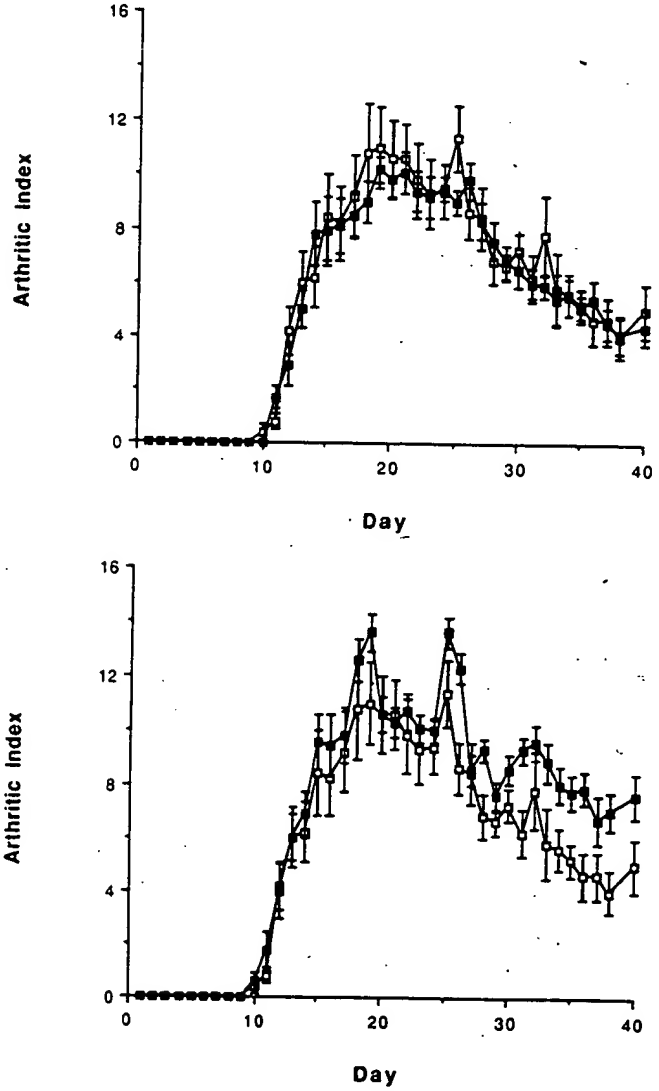


Figure 2. Impact of treatment with a non-relevant protein, human serum albumin, and IL-2 on induction of adjuvant arthritis. Animals were immunized with adjuvant on day 0 and treated subcutaneously on days −1 to 9 with either Tris-buffered saline (□) or 2 mg/kg human serum albumin (■) (A); Tris-buffered saline (□) or 0.125 mg/kg human IL-2 (■) (B). Values are mean ± SEM for 10 animals.

consistently showed extensive soft tissue swelling throughout the paw and ankle region (Table 1). This finding was accompanied by narrowing of the joint spaces and moderate new bone formation as shown by irregular areas of greater radiodensity in the metastarsal region. In contrast, radiographs of hind limbs from animals treated with $DAB_{486}IL\text{-}2$ during induction (days −1 to 9) showed only mild soft tissue swelling in the ankle region and minimal bone destruction or new bone formation. Summary data for 50 animals (25 controls and 25 $DAB_{486}IL\text{-}2$-treated rats) randomly selected from six separate studies is shown in Table 1. Statistical analysis of the radiographic scores for these two groups showed that animals treated with $DAB_{486}IL\text{-}2$ were graded significantly lower than were buffer controls (Pearson's χ^2 $p = 0.005$; Fisher's exact test $p = 0.01$ comparing the frequency of scores of 0 and 1 *versus* the frequency of scores of 2 and 3).

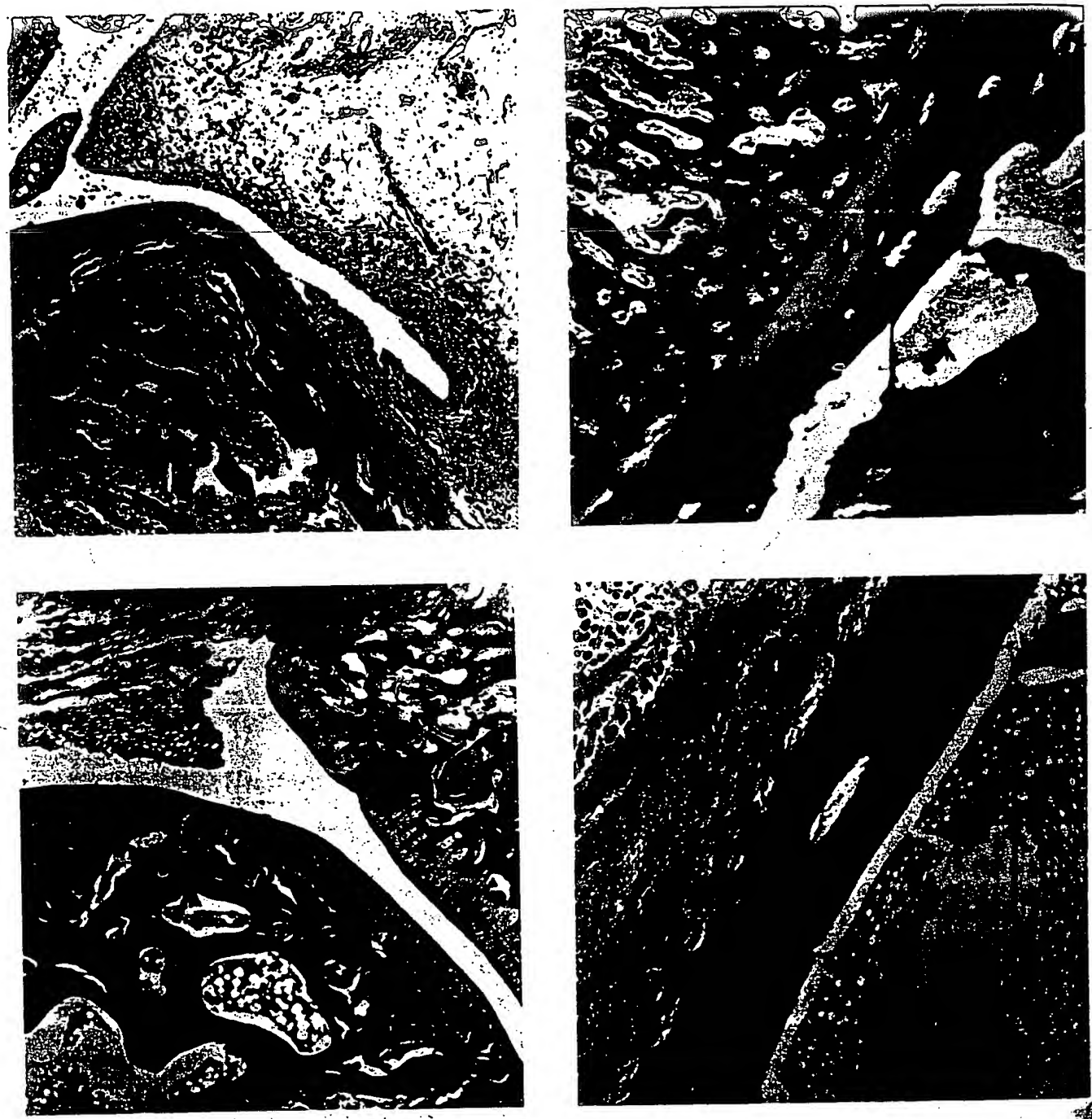


Figure 3. Decalcified ankle joint sections taken on day 22 from rats treated with buffer (A, B) or $DAB_{486}IL\text{-}2$ days −1 to 9 (C, D). Synovial thickening, moderate inflammatory infiltrate (II) and severe proliferative osteomyelitis characterized by extensive new bone (NB) formation perpendicular to and impinging on the existing bone can be seen in control animals. Minor thickening of the synovial membrane with mild inflammation is observed in animals treated with $DAB_{486}II\text{-}2$. New bone formation is greatly reduced in these animals. (hematoxylin and eosin stain, × 40)

Table 1. Summary of clinical, radiographic and histologic findings[a)]

	Buffer treated	$DAB_{486}IL$-2 treated
Arthritic index	6.5 ± 0.6	2.9 ± 0.2
Radiographic score	1.0 ± 0.2	0.5 ± 0.1
Histologic score	1.9 ± 0.2	0.8 ± 0.1

a) Values are mean score ± SEM of 25 animals for each group randomly selected from six separate studies. Blinded assessments were conducted as described in the Sects. 2.5, 2.6, 2.7 (arthritic index on a scale of 0 to 16, radiographic score and histologic score on scales of 0 to 3). Statistical analysis is described in the text.

3.3 Histological features

Microscopic examination of hematoxylin and eosin-stained paraffin-embedded sections of the joint in the hind paws of buffer-treated control animals, terminated on day 22 post-immunization, revealed extensive signs of disease. Findings included a thickened synovial joint lining, widespread inflammatory infiltrate forming granulomatous lesions and severe proliferative osteomyelitis characterized by bone destruction and new bone formation perpendicular to and impinging on the existing bone (Fig. 3A and B). Examination of the joints of animals treated with $DAB_{486}IL$-2 during the induction phase (day −1 to 9) indicated that $DAB_{486}IL$-2 treatment greatly reduced the infiltration of inflammatory cells and extensive bone remodeling characteristic of adjuvant arthritis, confirming the radiological results described above (Fig. 3C and D). Statistical analysis of the histographic scores for the two groups described above and summarized in Table 1 showed similar results to that for the radiographic scores, *i.e.* animals treated with $DAB_{486}IL$-2 were graded significantly lower than were buffer controls (Pearson's χ^2 $p = 0.0001$; Fisher's exact test $p = 0.0001$ comparing the frequency of scores of 0 and 1 *versus* the frequency of scores of 2 and 3).

3.4 Proliferative responses

The proliferative response of popliteal lymph node cells isolated from animals either 10 or 20 days postimmunization with mycobacterial adjuvant was assessed. Cells isolated from buffer-treated animals responded briskly to stimulation with *M. butryicum* (Table 2). In comparison, cells which were derived from $DAB_{486}IL$-2-treated animals and then stimulated with mycobacterial antigen incorporated significantly less [^{3}H] thymidine. This observation is not the result of general depression of the proliferative response since Con A stimulation of cells from $DAB_{486}IL$-2-treated animals was equivalent to that of cells derived from buffer-treated control animals.

3.5 Impact of preexisting antibodies to $DAB_{486}IL$-2

Active immunization programs against diphtheria have existed for more than 50 years in the developed world; thus, individuals may have levels of preexisting antibodies to diphtheria toxin which could potentially inhibit DAB_{486}-

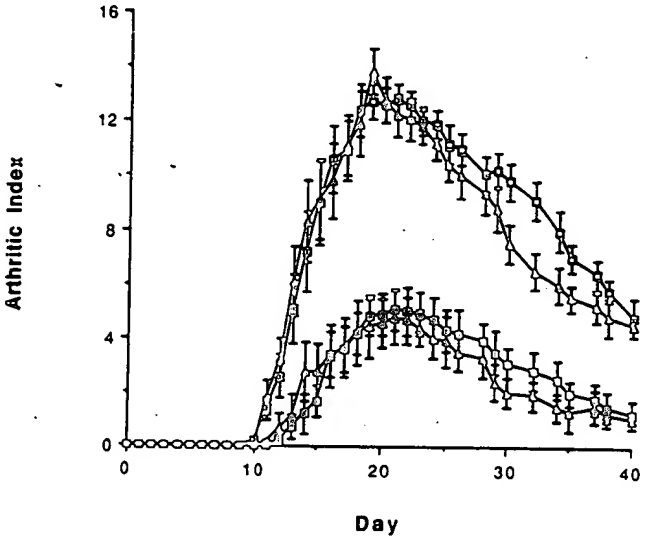


Figure 4. Impact of treatment with $DAB_{486}IL$-2 on induction of adjuvant arthritis in rats with preexisting antibodies to the diphtheria portion of the $DAB_{486}IL$-2 molecule. Half of the animals were preimmunized with diphtheria toxoid and developed moderate levels of anti-diphtheria toxin antibodies which cross-reacted with $DAB_{486}IL$-2 prior to immunization with adjuvant on day 0. Animals were treated with either Tris-buffered saline (naive, □; preimmune, ◇) or 0.5 mg/kg $DAB_{486}IL$-2 (naive, □; preimmune, ◇) on days −1 to 9. Values are mean ± SEM for 10 animals.

Table 2. Proliferative response of lymphocytes from popliteal lymph nodes to *M. butryicum* as measured by [^{3}H] thymidine uptake[a)]

Experiment Day	Adjuvant immunization	$DAB_{486}IL$-2 treatment	Culture conditions: Medium	Culture conditions: *M. butryicum*
10	+	−	2456 ± 391	14 976 ± 266
	+	+	3903 ± 565	61 845 ± 1018[b)]
20	+	−	2864 ± 301	32 633 ± 615[b)]
	+	+	2463 ± 171	4446 ± 449
	[illegible]	[illegible]	3500 ± 317	41 107 ± 3745
	[illegible]	[illegible]	2038 ± 975	8709 ± 6582[b)]

a) Values are mean cpm ± SEM of quadruplicate cultures of five animals each. See Sect. 2.8 for details.
b) $p<0.005$ *vs.* buffer-treated adjuvant-immunized animals (Student's *t*-test).

IL-2 activity *in vivo*. To evaluate the potential effect of anti-diphtheria toxin antibodies on $DAB_{486}IL$-2 activity, rats were immunized with diphtheria toxoid prior to immunization with adjuvant and $DAB_{486}IL$-2 treatment. At the time of immunization with *M. butryicum*, these animals had moderate levels of antibodies to diphtheria toxin and $DAB_{486}IL$-2 (average ELISA titer of 1:125 and a neutralizing antibody level of at least 0.01 Unit/ml). However, the presence of antibodies to diphtheria toxin did not alter the efficacy of treatment with $DAB_{486}IL$-2 during the induction phase of disease (Fig. 4). The clinical course for both naive and preimmune animals was identical. $DAB_{486}IL$-2-treated animals, both preimmune and naive, had a peak mean arthritic index of 4 while control animals, both preimmune and naive, had a peak mean arthritic index of 13.

3.6 Impact of delayed treatment with $DAB_{486}IL$-2

To investigate further the ability of $DAB_{486}IL$-2 to impact on the underlying bone destruction of adjuvant-induced arthritis, the effect of $DAB_{486}IL$-2 treatment during established disease was examined. Unlike the effect observed with $DAB_{486}IL$-2 treatment during the induction phase of disease, administration of the same dose of $DAB_{486}IL$-2 starting after arthritic symptoms had developed on day 11 and continuing until day 21, did not alter the measurable clinical signs of disease (Fig. 5). Histological sections of joints from rats which had received delayed treatment showed widespread mononuclear inflammatory infiltrate consistent with clinical signs of inflammation. However, radiographic analyses from three separate studies have shown that there was a trend towards a smaller percentage of animals with extensive bone erosion and new bone formation (grade 2 and 3) in the $DAB_{486}IL$-2-treated group (24%) than in the buffer-treated control group (53%), despite similar clinical arthritic indices ($p = 0.07$, Fisher's exact test).

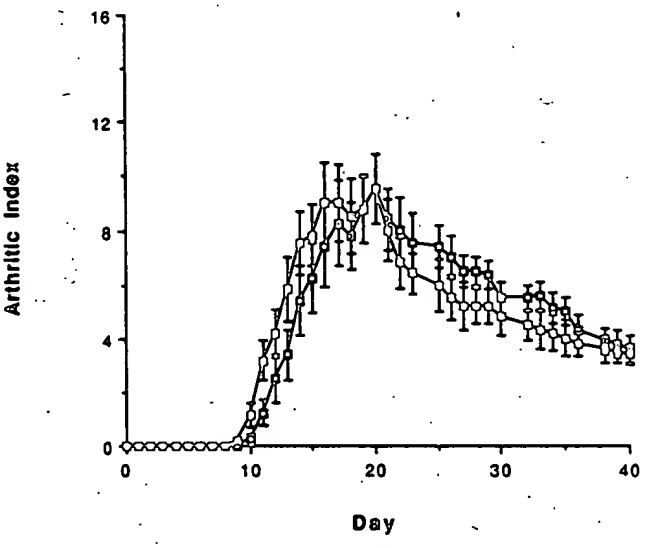


Figure 5. Impact of treatment with $DAB_{486}IL$-2 on existing adjuvant arthritis. Animals were immunized with adjuvant on day 0 and treated with either Tris-buffered saline (□) or 0.5 mg/kg $DAB_{486}IL$-2 (□) on days 11 to 21. Values are mean ± SEM for 10 animals.

4 Discussion

Rat adjuvant arthritis is a classic model of T lymphocyte-mediated autoimmune disease which shares many clinical and pathological similarities with rheumatoid arthritis. This model provides an excellent opportunity to assess the efficacy of T lymphocyte-targeted therapeutic approaches. We have shown that treatment of adjuvant arthritis with an IL-2 receptor-targeted cytotoxin during the induction phase of disease dramatically altered its clinical course by greatly reducing chronic inflammation and bone erosion. In addition, adjuvant-reactive cells were shown to be partially eliminated by $DAB_{486}IL$-2 treatment. Although treatment with $DAB_{486}IL$-2 at the onset of clinical symptoms of disease did not alleviate acute inflammation, there was a trend towards a smaller percentage of animals with extensive bone erosion and new bone formation in the $DAB_{486}IL$-2-treated group than in the buffer-treated control group. Since acute inflammation is characterized by local invasion of IL-2 receptor-negative cells such as neutrophils, it is understandable that $DAB_{486}IL$-2 treatment would have little effect on this feature of acute adjuvant arthritis. We are currently evaluating $DAB_{486}IL$-2 therapy in combination with anti-inflammatory agents in this model.

Two other groups have evaluated IL-2 receptor-targeted therapies in rat adjuvant arthritis. Stunkel et al. [14] have shown that treatment with ART-18, a monoclonal antibody to the rat low-affinity IL-2 receptor subunit, completely inhibited the passive transfer of adjuvant arthritis. However, ART-18 was incapable of controlling disease induced by adjuvant immunization. Another IL-2 receptor-specific agent, IL-2-PE40, lessened the severity of arthritis that developed when it was administered during the induction phase of disease [15, 16]; however, neither antigen-specific cytotoxicity nor delayed treatment was addressed in this study.

A major theoretical concern for the clinical use of $DAB_{486}IL$-2 is the presence of preexisting antibodies to diphtheria toxin in the human population which could potentially decrease the efficacy of $DAB_{486}IL$-2 treatment. Although several population studies have shown that titers of anti-diphtheria toxin antibodies are quite low in adults [17–19], the possibility exists that cross-reactive antibodies could neutralize the action of $DAB_{486}IL$-2. On the other hand, there are several reasons why such antibodies may not block intoxication of sensitive cells including the fact that anti-diphtheria toxin antibodies should not directly interfere with the binding of the IL-2 portion of the fusion toxin to the IL-2 receptor. In addition, the affinity of these antibodies for $DAB_{486}IL$-2 is much less than the affinity of the molecule for the IL-2 receptor. In the study presented here, immunization of rats with diphtheria toxoid prior to induction of adjuvant arthritis did not impair the efficacy of $DAB_{486}IL$-2. These rats had anti-diphtheria toxin antibody levels which are similar to those of healthy individuals. Clearly, measurement of antibody levels in a static *in vitro* system is not predictive of potentially more dynamic *in vivo* interactions.

$DAB_{486}IL$-2 has previously been shown to be selectively immunosuppressive in several murine model systems including delayed-type hypersensitivity and transplant

rejection [20–23]. To date there has been no evidence of general immunosuppression in animals treated with DAB_{486}IL-2 or in patients with IL-2 receptor-expressing malignancies receiving DAB_{486}IL-2 in phase I/II clinical trials. These attributes, together with the data reported here, provide support for the clinical evaluation of DAB_{486}IL-2 as a selective immunomodulator for the treatment of autoimmune diseases such as rheumatoid arthritis. Initial results from a phase I clinical trial in a small number of refractory rheumatoid arthritis patients have indicated that in contrast to currently available treatments, DAB_{486}IL-2 may be a fast acting well-tolerated anti-arthritic agent (D. Trentham, personal communication).

Received January 17, 1992; in revised form March 13, 1992.

5 References

1 Smith, K. A., *Science* 1988. *240:* 1169.
2 Waldmann, T. A., Goldman, C. K., Robb, R. J., Depper, J. M., Leonard, W. J., Sharrow, S. O., Bongiovanni, K. F., Korsmeyer, S. J. and Greene, W. C., *J. Exp. Med.* 1984. *160:* 1450.
3 Holter, W., Goldman, C. K., Casabo, L., Nelson, D. L., Greene, W. C. and Waldmann, T. A., *J. Immunol.* 1987. *138:* 2917.
4 Espinoza-Delgado, I., Ortaldo, J. R., Winkler-Pickett, R., Sugamura, K., Varesio, L. and Longo, D. L., *J. Exp. Med.* 1990. *171:* 1821.
5 Pearson, C. M. and Wood, F. D., *Arthritis Rheum.* 1959. *2:* 440.
6 Holoshitz, J., Klajman, A., Drucker, I., Capidot, Z., Yaretzki, A., Frenkel, A., van Eden, W. and Cohen, I. R., *Lancet* 1986. *ii:* 305.
7 Prud'homme, G. J. and Parfey, N. A., *Lab. Invest.* 1988. *59:* 158.
8 Bacha, P., Williams, D. P., Waters, C., Williams, J. M., Murphy, J. R. and Strom, T. B., *J. Exp. Med.* 1988. *167:* 612.
9 Waters, C. A., Schimke, P. A., Snider, C. E., Itoh, K., Smith, K. A., Nichols, J. C., Strom, T. B. and Murphy, J. R., *Eur. J. Immunol.* 1990. *20:* 785.
10 Walz, G., Zanker, B., Brand, K., Waters, C., Genbauffe, F., Zeldis, J. B., Murphy, J. R. and Strom, T. B., *Proc. Natl. Acad. Sci. USA* 1989. *86:* 9485.
11 Williams, D. P., Parker, K., Bacha, P., Bishai, W., Borowski, M., Genbauffe, F., Strom, T. B. and Murphy, J. R., *Protein Eng.* 1987. *1:* 493.
12 Trentham, D. E., Townes, A. S. and Kang, A. H., *J. Exp. Med.* 1977. *146:* 857.
13 Popovic, M., Lange-Wantzin, G., Sarin, P., Mann, S. D. and Gallo, R. C., *Proc. Natl. Acad. Sci. USA* 1983. *8:* 5402.
14 Stunkel, K. G., Theisen, P., Mouzaki, A., Diamantstein, T. and Schlumberger, H. D., *Immunology* 1988. *64:* 683.
15 Case, J. P., Lorberboum-Galski, H., Lafyatis, R., FitzGerald, D., Wilder, R. L. and Pastan, I., *Proc. Natl. Acad. Sci. USA* 1989. *86:* 287.
16 Lorberboum-Galski, H., Lafyatis, R., Case, J. P., FitzGerald, D., Wilder, R. L. and Pastan, I., *J. Immunopharmacol* 1991. *13:* 305.
17 Crossley, K. P., Warren, J. B., Lee, B. L. and Mead, K., *JAMA* 1979. *242:* 2298.
18 Sargent, R. K., Rossing, T. H., Dowtor, S. B., Breyer, M. D., Levine, L. and Weinstein, L., *Am. J. Med. Sci.* 1984. *287:* 37.
19 Kjeldsen, K., Simonsen, O. and Heron, I., *Lancet* 1985. *i:* 900.
20 Kelley, V. E., Bacha, P., Pankewycz, O., Nichols, J. C., Murphy, J. R. and Strom, T. B., *Proc. Natl. Acad. Sci. USA* 1988. *85:* 3880.
21 Pankewycz, O., Mackie, J., Hassarjian, R., Murphy, J. R., Strom, T. B. and Kelley, V. E., *Transplantation* 1989. *47:* 318.
22 Kirkman, R. L., Bacha, P., Barrett, L. V., Forte, S., Murphy, J. R. and Strom, T. B., *Transplantation* 1989. *47:* 327.
23 Bastos, M. G., Pankewycz, O., Rubin-Kelley, V. E., Murphy, J. R. and Strom, T. B., *J. Immunol.* 1990. *145:* 3535.

A Human Immunodeficiency Virus–Transgenic Mouse Model for Assessing Interventions that Block Microbial-Induced Proviral Expression

Marco L. Schito,[1] Paul E. Kennedy,[2] Rachel P. Kowal,[1] Edward A. Berger,[2] and Alan Sher[1]

[1]*Immunobiology Section, Laboratory of Parasitic Diseases, and* [2]*Molecular Structure Section, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland*

A human immunodeficiency virus (HIV) type 1–transgenic mouse line (166) that previously showed up-regulated expression of viral proteins and infectious particles after infection with pathogenic agents was tested as a model for screening the in vitro and in vivo efficacy of inhibitors of HIV-1 immune activation. Two types of interventions were assessed: use of either the immunosuppressive drug prednisolone or an HIV-1 envelope–targeted toxin (sCD4-PE40). Both agents inhibited lipopolysaccharide-induced p24 expression by splenocytes in vitro and, when administered to transgenic mice, suppressed the induction of plasma p24, as well as the ex vivo production of p24 and infectious virus stimulated by in vivo infection with *Mycobacterium avium*. Moreover, HIV-1 mRNA levels in the spleen were greatly reduced in mice treated with either agent. Because HIV-1 expression cannot be induced in T lymphocytes from line 166 mice, this model may be of particular advantage for testing interventions that target virus production by non–T cell virus reservoirs.

A major problem in the treatment of human immunodeficiency virus (HIV) type 1 infection is the existence of latently infected cells that constitute reservoirs for the re-emergence of active viral replication. Such reservoirs have been localized to both CD4 T cell populations and macrophage and monocyte cell populations and persist in the face of effective highly active antiretroviral therapy (HAART) [1–3]. Although not well understood, the reactivation of latent HIV-1 appears to be influenced strongly by immunologic stimuli, including microbial coinfections [4–8] and vaccination [9–11]. Thus, the development of interventions that specifically block latent virus resulting from immune activation may be an important strategy both for reducing HIV-1 load during active infection and for long-term control of viral latency.

Several agents have been tested for their ability to inhibit HIV-1 immune activation both in vitro and in limited clinical trials. These include immunosuppressive drugs (such as glucocorticoids [12–14] and thalidomide [15, 16]), retinoic acid [17, 18], cytokines (e.g., interferon-α and interleukin [IL]–13) [19–22], and antagonists of cytokines (e.g., IL-1, tumor necrosis factor [TNF]–α, and macrophage colony-stimulating factor) [23–25]. A basic problem encountered in previous studies, particularly during in vivo trials, was the inability to directly assess whether the intervention tested targets the activation of integrated virus.

We studied a transgenic (Tg) mouse model of HIV-1 immune activation that offers an approach for screening and identifying inhibitors that specifically suppress proviral induction. Tg mice carrying intact or internally deleted HIV-1 DNA sequences have been used by several investigators to analyze factors involved in AIDS pathogenesis and in the control of viral gene expression [26–35]. We used Tg mouse line 166, which incorporates the entire HIV-1 genome (including the long terminal repeat [LTR]) of the NL4-3 molecular clone inserted in multiple copies at a single integration site [30, 36]. Line 166 Tg mice constitutively express low levels of viral RNA and proteins [36, 37] and, in contrast to other Tg mice that carry internally deleted HIV sequences [27–34], fail to display identifiable immunological defects or significant disease. When exposed either in vitro or in vivo to microbial stimuli, such as *Toxoplasma gondii, Mycobacterium avium,* or *Escherishia coli* endotoxin, cells from these mice up-regulate their expression of HIV-1–specific genes, p24 core protein, and infectious virus, as detected by coculture with human T cells [36, 37]. In the line 166 Tg mouse model, antigen-presenting cells—such as monocytes and macrophages [37], dendritic cells, and B cells [38, 39]—rather than T lymphocytes appear to be the major sources of viral expression. There is growing evidence that antigen-presenting cells can serve as important virus reservoirs in infected humans [40]. Since no lateral spread of HIV-1 infection can occur in

Received 4 January 2001; revised 1 March 2001; electronically published 1 May 2001.

Presented in part: Keystone Symposium on HIV Pathogenesis, Keystone, Colorado, April 2000 (abstract 151).

All animal experimentation was done under guidelines of the National Institute of Allergy and Infectious Diseases in an escape-proof animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Financial support: National Institutes of Health Intramural AIDS Targeted Antiviral Program.

Reprints or correspondence: Dr. Marco Schito, LPD, NIAID, 4 Center Dr., Rm. 4/132, National Institutes of Health, Bethesda, MD 20892-0425 (mschito@niaid.nih.gov).

The Journal of Infectious Diseases 2001; 183:1592–600

0022-1899/2001/18311-0006

line 166 Tg mice because of the absence of the required human CD4 and coreceptor molecules, these mice also offer a means for studying the events involved in immune activation of integrated virus in isolation from those required for infection and propagation.

In the present study, we assessed the utility of the line 166 Tg mouse as a model for screening agents that inhibit microbial-induced HIV-1 expression. To do so, we tested 2 inhibitors described elsewhere [12, 14, 42, 43], first for their ability to block endotoxin (lipopolysaccharide [LPS])–induced p24 expression from Tg splenocytes and then for their suppression of in vivo p24 production stimulated by short-term infection of the mice with a virulent strain of *M. avium*. The 2 agents examined have different modes of action. The first, the glucocorticoid drug prednisolone, has been shown elsewhere to suppress HIV-1 LTR activation by cytokine-stimulated transfected promonocytic cells in vitro [14]. AIDS patients treated with prednisolone for ≤1 year displayed transient increases in CD4 cell counts and stabilized virus loads [12]. The effects of glucocorticoids on HIV-1 have been attributed to their known inhibition of NF-κB– and/or AP-1–dependent transcriptional events [13, 41]. The second agent, the envelope glycoprotein (*env*)–targeted toxin soluble CD4 (sCD4)–PE40, is a genetically engineered chimeric protein containing the gp120-binding region of human CD4 linked to the translocation and ADP-ribosylation domains of *Pseudomonas aeruginosa* exotoxin A [42]. This hybrid toxin binds selectively to HIV-1–infected cells and is translocated into the cytoplasm, where it inhibits protein synthesis, which results in cell death. Previous in vitro studies have demonstrated the efficacy of sCD4-PE40 in selectively killing HIV-1–infected cells and in limiting virus spread [43]. Moreover, the chimeric toxin has been shown in vivo to enhance the capacity of HAART to suppress acute and chronic HIV-1 infection in a model using SCID mice with human fetal thymus and liver transplants [44]. The in vitro and in vivo effects of prednisolone and the chimeric toxin sCD4-PE40 on microbial-induced viral expression in HIV-1–Tg mice are described below.

Materials and Methods

HIV-1–Tg mice. Tg mouse line 166 was derived by transfecting the full-length wild-type NL4-3 viral clone into FVB/N mice, as described elsewhere [30, 36]. The mice have ~20–60 copies of the transgene at a single integration site and transmit them in a stable Mendelian fashion. Tg mice were maintained by homozygous and heterozygous breeding under specific pathogen–free conditions in an escape-proof room within the animal care facilities of the National Institute of Allergy and Infectious Diseases (NIAID, Bethesda, MD). Non-Tg FVB/N control mice were purchased from Division of Cancer Treatment/National Cancer Institute and were housed in the same room as the Tg mice. Age- and sex-matched Tg and control mice 8–12 weeks old were used in all experiments.

HIV-1 inhibitors and controls. For in vitro studies, prednisolone (Sigma, catalog number P-6004) was dissolved in ethanol and was used at a final concentration of 0.25, 2.5, or 25 ng/mL. Control-treated wells contained ≤0.04% ethanol, which did not alter the production of HIV-1 p24 from cultured spleen cells. For in vivo treatment, prednisolone 21-hemisuccinate (Sigma, catalog number P-4153) was used because of its water-soluble properties, thus eliminating possible confounding effects of the ethanol vehicle. The glucocorticoid was administered orally at 30 mg/kg/day, a dose shown elsewhere to completely suppress *Borrelia burgdorferi*–induced arthritic joint swelling in mice [45].

The sCD4-PE40 and sCD4 molecules tested were donated by S. Johnson (Pharmacia-Upjohn). The CD4 portions of both molecules contain the first 2 extracellular domains. The PE40 portion contains the translocation and the cell-killing domains genetically engineered from *P. aeruginosa* exotoxin A. The molecules were used at 1–100 n*M* for in vitro studies. Mice were injected intraperitoneally (ip) with a dose of 40 μg/kg mouse weight of either sCD4-PE40 or control sCD4 every other day for a total of 6 injections.

Assay for inhibition of HIV-1 expression in vitro. Spleens from 2–5 uninfected Tg mice were disrupted through a nylon mesh, to obtain single-cell suspensions that were pooled and stimulated with 100 ng/mL of *E. coli* 0128:B12 LPS (Sigma, catalog number L-2755), in the presence of the indicated inhibitors for the designated times. Controls included cells not stimulated with LPS or not treated with inhibitors. The cultures (5×10^6 cells/mL) were incubated at 37°C with 5% CO_2 in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum (HyClone), 10 m*M* HEPES (Life Technologies), 2 m*M* glutamine (National Institutes of Health [NIH] stock), 100 U/mL penicillin, 100 μg/mL streptomycin (NIH stock), and 5.5×10^{-5} *M* β-2-mercaptoethanol (Life Technologies) (hereafter referred to as "complete medium"). Supernatants were removed daily for a 4-day culture period and were stored frozen at −20°C.

In vivo assay for inhibition of HIV-1 expression induced by M. avium. To test the effect of inhibitors in vivo, we used a model in which Tg provirus was induced as a consequence of experimental infection with *M. avium*. The *M. avium* strain 2-151 SmT was obtained as a gift from A. Cooper (Colorado State University, Fort Collins). Bacterial stocks containing 10^9–10^{10} cfu bacteria/mL in PBS were prepared and stored frozen at −70°C [46]. Mice were infected by intravenous injection of 10^8 cfu bacteria resuspended in a total volume of 200 μL PBS, and inhibitor treatment was initiated immediately after infection.

To assay p24 production in vivo, mice were bled from the tail vein 1 day before *M. avium* infection and also after termination of the experiment (day 11 after infection), by cardiac puncture, into EDTA-treated vacutainer tubes (Beckton Dickinson). Plasma samples were separated, were stored individually, and were frozen at −20°C. To determine p24 and HIV-1 production ex vivo, spleen cells from the Tg mice were cultured individually in complete medium containing 15 μg/mL ampicillin (Life Technologies), because penicillin and streptomycin are bacteriostatic for *M. avium*. Supernatants were removed after 24 h and were stored frozen at −20°C. To determine bacteria load, spleen cells isolated from mice were diluted serially, from 10^6 to 10^2 cells/mL, in PBS and were cultured in duplicate on Middlebrook agar plates, as described elsewhere [46]. The colony-forming units per spleen were calculated by multiplying the number of colony-forming units per 10^6 cells by the total number of cells in the spleen.

Quantitation of viral protein (p24) production. The HIV-1 p24 nucleocapsid antigen was quantified by using a commercial ELISA kit (Beckman-Coulter), according to the manufacturer's instructions. Supernatants from in vitro or ex vivo spleen cell cultures were diluted in complete medium, and p24 levels were determined in duplicate. Antigenemia was calculated as the *x*-fold increase in plasma p24 on the day the experiment was terminated versus the preinfection level for each mouse, to control for p24 variability among individual mice.

Quantitation of HIV-1 gene expression in vivo by real-time reverse transcriptase (RT)–polymerase chain reaction (PCR). Total RNA was isolated from portions (~25%) of spleen tissue by homogenization in 1 mL RNA STAT-60 (Tel-Test), using a tissue polytron (Omni International), as recommended by the manufacturer. The resulting RNA samples were resuspended in diethylpyrocarbonate-treated water and were quantitated spectrophotometrically. Single-strand cDNA was synthesized using the superscript preamplification system (Life Technologies). Amplification of *env*, *gag*, and *HPRT* cDNA was monitored with the fluorescent DNA-binding dye SYBR Green (Roche Diagnostics), using the LightCycler version 3 software system (Roche Diagnostics), according to the manufacturer's instructions. The following primer pairs were used: for *HPRT*, GTTGGTTACAGGCCAGACTTTGTTG (forward) and GAGGGTAGGCTGGCCTATAGGCT (reverse); for HIV *env*, GGGGACCAGGGAGAGCATT (forward) and TGGGTCCCCTCCTGAGGA (reverse); and for HIV *gag*, CCAGATGAGAGAACCAAGGG (forward) and TTGTGAAGCTTGCTCGGCTCT (reverse). LPS-stimulated splenocyte cDNA was used to generate standard curves for each mRNA of interest. The results were calculated as the level of *env* or *gag* gene expression normalized to the *HPRT* control values for each sample. Three repeat *env*, *gag*, and *HPRT* reactions were done for each sample, and an aggregate mean ± SD was determined for each animal group.

Assay for infectious virions. The multinuclear activation of a galactosidase indicator (MAGI) assay was used to detect replication-competent virus particles, as described elsewhere [47]. In brief, the CD4-LTR/β-galactosidase–transfected HeLa cells (provided to M. Martin, NIAID, by M. Emerman, Fred Hutchinson Cancer Research Center, Seattle) were plated at a density of 4×10^4 cells/well in 24-well plates in complete medium 1 day before assay. The next day, spent medium was removed from the wells and was replaced with 100 μL or 10 μL of spent medium from experimental and control spleen cells cultured ex vivo for 24 h; the wells then were brought to a final volume of 180 μL with fresh culture medium. DEAE-dextran (20 μL of a 200 μg/mL stock) also was added to each well. After 2 h at 37°C, 1 mL of warm complete medium was added, and the cultures were incubated for an additional 48 h. Cells then were washed with PBS, were fixed with 1% formaldehyde and 0.2% glutaraldehyde, and were stained with 4 m*M* potassium ferrocyanide, 4 m*M* potassium ferrocyanide, 2 m*M* $MgCl_2$, and 0.4 mg X-Gal/mL for 50 min at 37°C. Blue cells were counted using an inverted microscope at a final magnification of ×100. Data are expressed as the number of β-galactosidase–positive cells in a total of 4 different fields.

Statistical analysis. Student's *t* test was used to evaluate the statistical significance between experimental and control Tg mice.

Results

Ability of agents to block LPS-induced p24 production in vitro. Our previous study has shown that HIV-1 p24 expression is significantly up-regulated when spleen cells from line 166 Tg mice are cultured in the presence of LPS [37]. We used this in vitro assay to pretest the efficacy of the 2 candidate inhibitors of HIV-1 activation examined in the present study. Single-cell suspensions of splenocytes were cultured with a fixed dose of LPS in the presence of increasing concentrations of each agent, and supernatants were assayed for p24 at 72 h, a time point at which production of this HIV-1 protein increases exponentially in the absence of inhibitors.

The glucocorticoid drug prednisolone suppressed, in a dose-dependent manner, LPS-induced p24 production in spleen cells

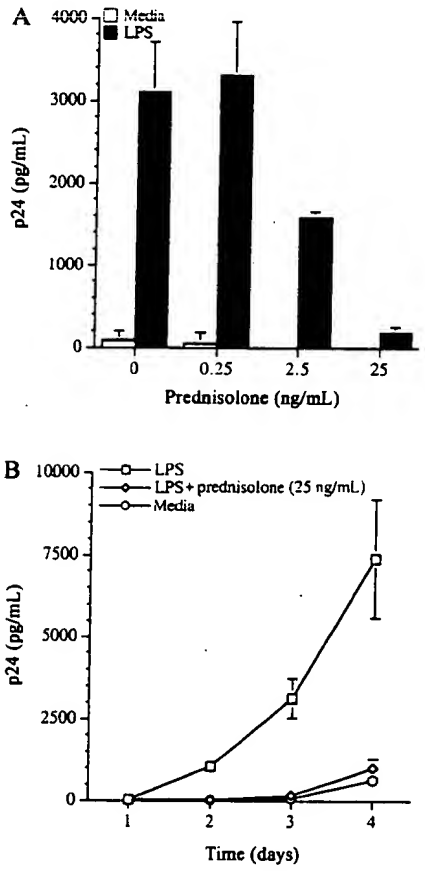


Figure 1. Inhibition by prednisolone of lipopolysaccharide (LPS)–stimulated p24 protein expression by spleen cells from human immunodeficiency virus type 1–transgenic mice. *A*, Dose response. Cells were incubated with or without LPS (100 ng/mL), in the presence of the indicated concentrations of prednisolone, and supernatants were assayed for p24 at 72 h. *B*, Time course. Splenocytes were incubated in complete medium alone or stimulated with LPS in the presence or absence of prednisolone (25 ng/mL), and supernatants were assayed for p24 at the indicated times. Data represent the means of assays on triplicate wells, with error bars indicating the SDs. The results shown are representative of 2 experiments performed.

from Tg mice, with 25 ng prednisolone/mL causing nearly complete inhibition (figure 1*A*) throughout the entire time course (figure 1*B*). No effect of the ethanol vehicle was observed in the same assay (data not shown). The observed suppression was not due to cell toxicity, since the doses examined did not significantly affect macrophage, T cell, or B cell viability in LPS-stimulated cultures, as determined by propidium iodide staining and flow cytometry (data not shown).

Dose-dependent inhibition of LPS-stimulated p24 production also was observed with the *env*-targeted toxin sCD4-PE40, with 100 n*M* sCD4-PE40 causing nearly complete suppression of p24 production, compared with that seen in unstimulated cells (figure 2*A*). No effect of unconjugated sCD4 was observed at the same dose, thus demonstrating that the activity of the chimeric toxin is not simply due to binding of HIV-1–expressing cells to the toxin's CD4 moiety and requires the toxin portion of the molecule. Moreover, the inhibitory activity of sCD4-PE40 was abrogated in a dose-dependent fashion by the addition of sCD4, with nearly complete competition achieved at 100-fold excess (figure 2*B*). The latter observation confirms that the effect of the toxin is dependent on interaction between the CD4 moiety and *env* expression on the stimulated cells and is not due to nonspecific cell killing. Nevertheless, we were unable to detect significant cell death in the toxin-treated splenocyte cultures (data not shown), by means of an MTT oxidation assay used in previous studies with sCD4-PE40 [48]. This observation, however, is consistent with previous findings that indicate that the percentage of HIV-1–expressing cells present in the spleen after microbial activation is quite low [37] and, thus, is perhaps too low to be revealed in a direct cell-killing assay.

Effects of agents on microbial-induced activation of HIV-1 expression in vivo. To assay prednisolone or sCD4-PE40 for its ability to block the activation of HIV-1 transgene expression in vivo, we used a model of *M. avium* infection in which bacterial infection stimulates p24 production that is detectable both in plasma and in ex vivo splenic cultures [37]. In the protocol used, mice were infected intravenously with *M. avium* (10^8 cfu/mouse) several hours after the initial administration of the agent, and p24 levels in plasma and spleen were measured on day 11 after infection.

To test the in vivo effects of prednisolone, *M. avium*–infected Tg mice were treated with the drug perorally on a daily basis for the entire period of the experiment. Prednisolone administration resulted in a marked inhibition of the splegomegaly induced by bacterial infection, which is consistent with its known anti-inflammatory effects (figure 3*A*). Furthermore, prednisolone-treated infected mice harbored significantly more bacteria in their spleens than did untreated infected mice (figure 3*B*). Despite the accompanying increase in bacteria load, both plasma p24 antigenemia (figure 3*C*) and ex vivo production of p24 by spleen cells (figure 3*D*) were suppressed significantly in the infected mice treated with prednisolone.

To test the in vivo effects of the *env*-targeted toxin sCD4-PE40, *M. avium*–infected Tg mice were given the recombinant molecule ip every 48 h, beginning on the day of infection. In contrast with the glucocorticoid-treated mice, *M. avium*–infected mice given the chimeric toxin showed no loss in spleen

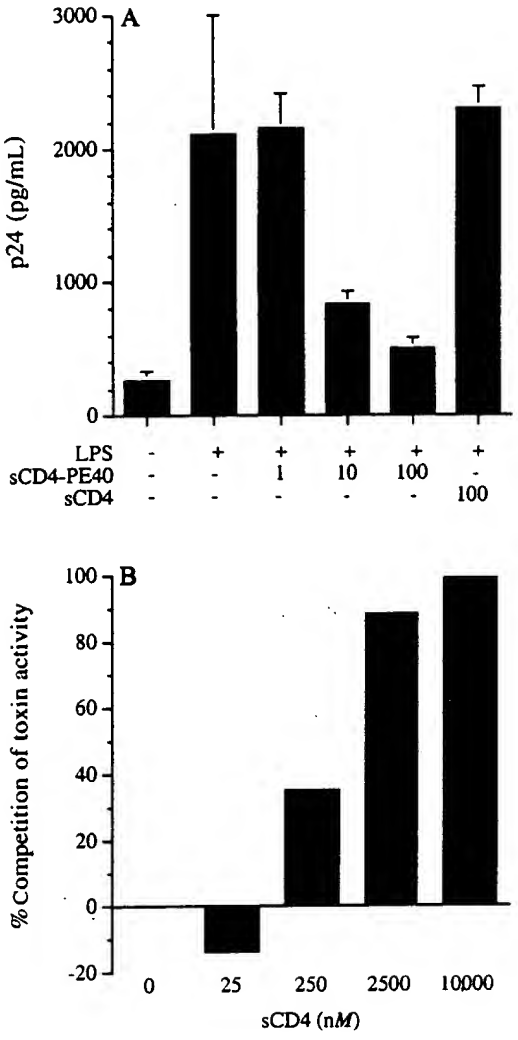


Figure 2. Inhibition by envelope glycoprotein–targeted toxin (soluble CD4 [sCD4]–PE40) of lipopolysaccharide (LPS)–stimulated p24 protein expression in spleen cells from human immunodeficiency virus type 1–transgenic (Tg) mice. *A*, Effects of sCD4-PE40 vs. unconjugated sCD4. Spleen cells from Tg mice were cultured in the presence of LPS (100 ng/mL) and the indicated concentrations of sCD4-PE40 (in n*M*) or sCD4 (in n*M*). *B*, Competition by sCD4 of sCD4-PE40–mediated inhibition of p24 production. Spleen cells were cultured in the presence of LPS (100 ng/mL) and sCD4-PE40 (100 n*M*) plus the indicated concentrations of sCD4. Results are percentage competition by sCD4, with 0% competition defined as the level of p24 measured in cultures containing sCD4-PE40 in the absence of sCD4 and 100% competition defined as the level induced by LPS in the absence of sCD4-PE40. In both *A* and *B*, supernatants were collected at 72 h for p24 analysis (parallel results were obtained using 24-, 48-, and 96-h time points). Data represent the means of assays on duplicate wells, with error bars indicating SDs. The results shown are representative of 3 experiments performed.

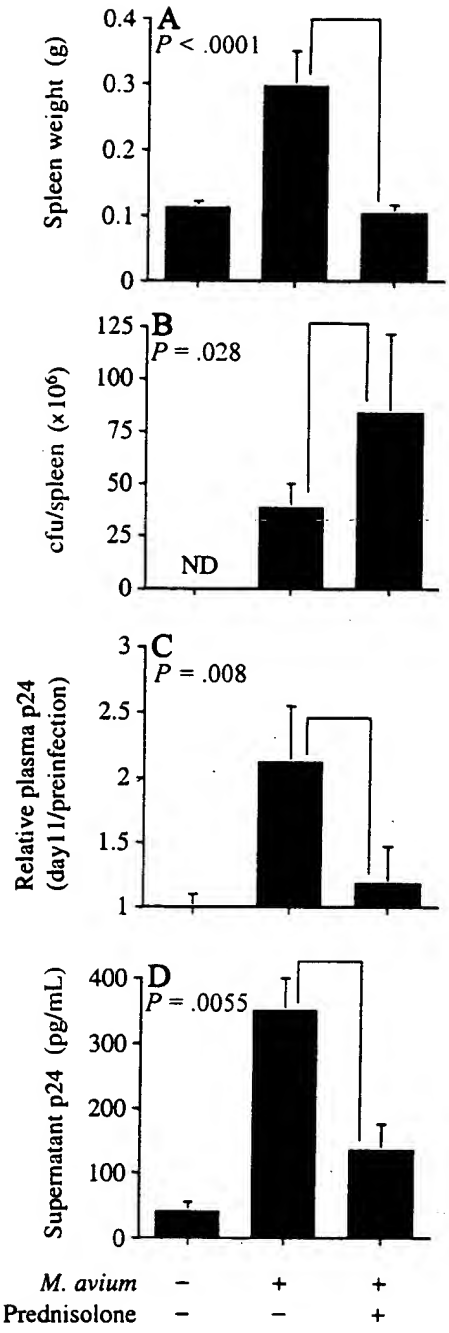


Figure 3. Inhibition by prednisolone by *Mycobacterium avium*–induced human immunodeficiency virus type 1 p24 expression in vivo, despite enhanced bacterial replication. Starting at the time of infection, *M. avium*–exposed mice ($n = 4$) were treated daily with prednisolone (30 mg/kg/day, orally) or were left untreated ($n = 6$). A third group ($n = 3$) consisted of age-matched, uninfected, untreated mice. Mice were killed 11 days after infection and were analyzed individually, for spleen weight *(A)*, bacterial colony-forming units per spleen *(B)*, plasma p24 levels at day 11 relative to day 0 ("preinfection") for each mouse *(C)*, and ex vivo production of p24 by spleen cell cultures *(D)*, within 24 h. Data represent the means for each group of mice, with error bars indicating the SDs. The results shown are representative of 2 experiments performed.

weight (figure 4*A*) or significant alterations in splenic bacteria load (figure 4*B*) with respect to either infected mice treated with sCD4 or untreated infected control mice. Nevertheless, sCD4-PE40 significantly inhibited the *M. avium*–mediated activation of p24 production, as measured in vivo in plasma (figure 4*C*) or ex vivo in spleen cells (figure 4*D*). This effect clearly was dependent on the toxin component of the molecule, because sCD4 failed to alter p24 expression when given in the same regimen. We also observed that sCD4-PE40 had no effect on the background expression of p24 in Tg mice not infected with *M. avium* (data not shown).

To confirm that the suppressed ex vivo expression of p24 resulting from prednisolone or sCD4-PE40 treatment reflects decreased viral gene expression in situ, *env* and *gag* mRNA levels were quantitated by real-time RT-PCR in spleen samples from control and drug-treated mice. As shown in figure 5, *M. avium* infection results in a >2-fold increase in splenic *env* and *gag* messages in Tg mice. Treatment with prednisolone or sCD4-PE40, but not sCD4, reduced the expression of mRNA to the levels seen in uninfected mice.

Correlation between inhibition of activation of p24 and infectious virus production. To determine whether the reductions in p24 production observed in vivo after prednisolone or sCD4-PE40 treatment reflect suppressed production of infectious virions, we used HeLa cells transfected with CD4 and an LTR–β-galactosidase reporter gene (MAGI assay) [47]. This assay detects the early steps in the replication cycle of the virus that lead to production of Tat and to the resulting activation of the integrated *Lac-z* gene, which is linked to the HIV-1 LTR. Low numbers of positive cells were observed when the MAGI cultures were exposed to splenocyte supernatants from Tg mice not infected with *M. avium*. Although larger, these numbers were not significantly different from the background values detected using spleen-cell supernatants from non-Tg mice (FVB/N, with or without *M. avium* infection). *M. avium* infection of Tg mice resulted in a >4-fold increase in the number of β-galactosidase–positive cells detected in the assay (figure 6). This finding confirms and extends previous data that demonstrated increased RT activity in a human T cell line (MT4) after incubation with spleen cells from *M. avium*–infected Tg mice [37]. Together, both observations support the conclusion that small numbers of infectious particles can be assembled in Tg mice and that this process is up-regulated in response to microbial stimuli.

Prednisolone treatment blocked the enhancement of infectious virion production resulting from *M. avium* infection to the level seen in uninfected mice (figure 6). Treatment of *M. avium*–infected mice with sCD4-PE40 caused a lesser, but nevertheless highly significant, reduction in viral activity, relative to that measured in untreated or control sCD4-treated infected mice. Our results show that the inhibition of microbial-stimulated p24 synthesis induced by these 2 interventions reflects changes in the production of infectious virions.

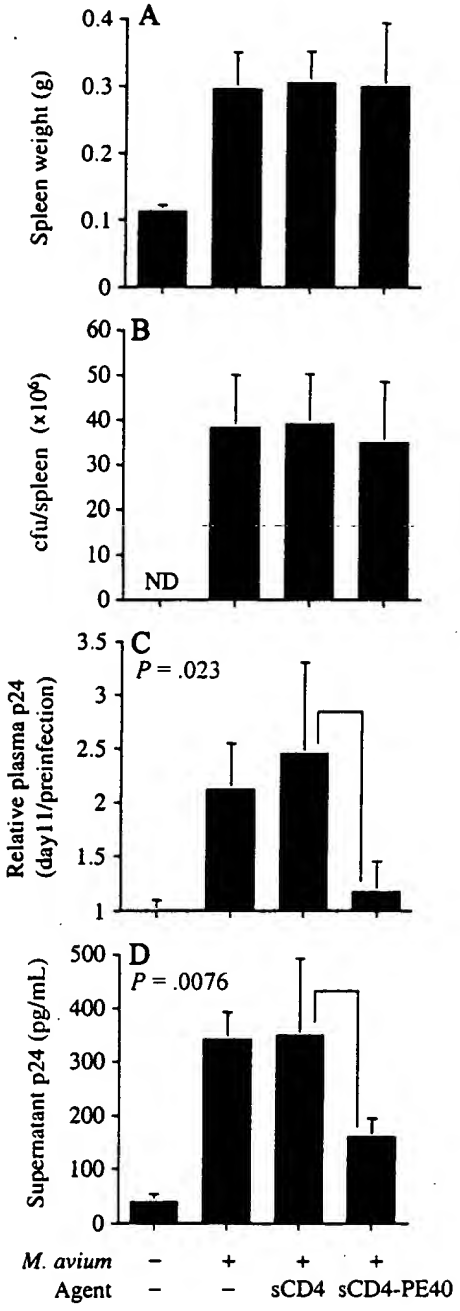


Figure 4. Inhibition by envelope glycoprotein–targeted toxin (soluble CD4 [sCD4]–PE40) of *Mycobacterium avium*–induced human immunodeficiency virus type 1 p24 expression in vivo. Starting at the time of infection, *M. avium*–exposed mice were injected intraperitoneally every other day with sCD4 ($n = 4$) or with sCD4-PE40 ($n = 6$) at 40 μg/kg of w[illegible]ht or were left untreated ($n = 3$). A fourth group ($n = 3$) consisted of a[illegible] matched, uninfected, untreated animals. Mice were killed 11 days after infection and were analyzed individually, for spleen weight *(A)*, bacterial colony-forming units per spleen *(B)*, plasma p24 levels at day 11 relative to day 0 ("preinfection") for each mouse *(C)*, and ex vivo production of p24 by spleen cell cultures *(D)*, within 24 h. Data represent the means for each group of mice, with error bars indicating the SDs. The results shown are representative of 2 experiments performed.

Discussion

The findings reported here establish the line 166 Tg mouse model as a useful tool for screening immunologic and pharmacologic interventions that inhibit the activation of integrated HIV-1. In the protocols that we have described, agents can be tested first for their suppression of endotoxin-induced p24 secretion by Tg spleen cells. They then can be assessed for their ability to block in vivo induction of either p24 or infectious virus by a secondary pathogenic agent—in this case, *M. avium*. A major advantage of this Tg mouse model is that inhibitors of HIV-1 activation can be studied without accounting for complications associated with spread of the viral infection, since mice lack the essential functional primary receptor (human CD4) and coreceptors (human CC chemokine receptor 5 and/or CXC chemokine receptor 4) required for HIV-1 entry.

To demonstrate the utility of our model, we tested 2 distinct types of interventions that attack different targets in the induction of integrated virus. The first intervention, the glucocorticoid drug prednisolone, is an anti-inflammatory agent that is likely to inhibit HIV-1 expression indirectly through its suppression of the immune response induced by LPS or *M. avium* microbial stimuli. By inhibiting the microbial-induced production of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, glucocorticoids thus could suppress viral induction. Although prednisolone can suppress transcription factors involved in inflammation, other effects, such as an increase in anti-inflammatory gene transcription and histone deacetylation [41], may contribute to the inhibition of the HIV-1 LTR promotor. Moreover, in vitro studies have demonstrated direct effects of these drugs on NF-κB–dependent transcriptional activation of the HIV-1 LTR [14, 23]. In our experiments, prednisolone clearly suppressed LPS-induced p24 production in vitro by a mechanism that did not involve cell death. The immunosuppressive effects of the drug were evident in vivo from the decreased weight of the spleen and increased bacteria load in the treated mice. Nevertheless, because prednisolone-injected mice showed increased rather than decreased bacteria counts, the suppression of in vivo virus levels in the spleen and plasma cannot be attributed to an indirect effect on host resistance to the *M. avium* infection. Although the precise mechanism of action of prednisolone was not addressed in the present study, our experiments clearly demonstrated the ability of our model to discriminate between the effects of immunopharmacologic inhibitors on immune activation of HIV-1 and immunologic control of the inducing pathogen itself.

sCD4-PE40, the second intervention tested for its effects on line 166 Tg mice, was specifically directed at HIV-1–expressing cells rather than at a host pathway involved in viral induction. This *env*-targeted chimeric toxin was highly active in blocking LPS-induced p24 production. The lack of activity of unconjugated sCD4 in the same assay suggests that the reductions in p24 expression induced by sCD4-PE40 are due to toxin-mediated killing of HIV-1–expressing cells. Moreover, the ef-

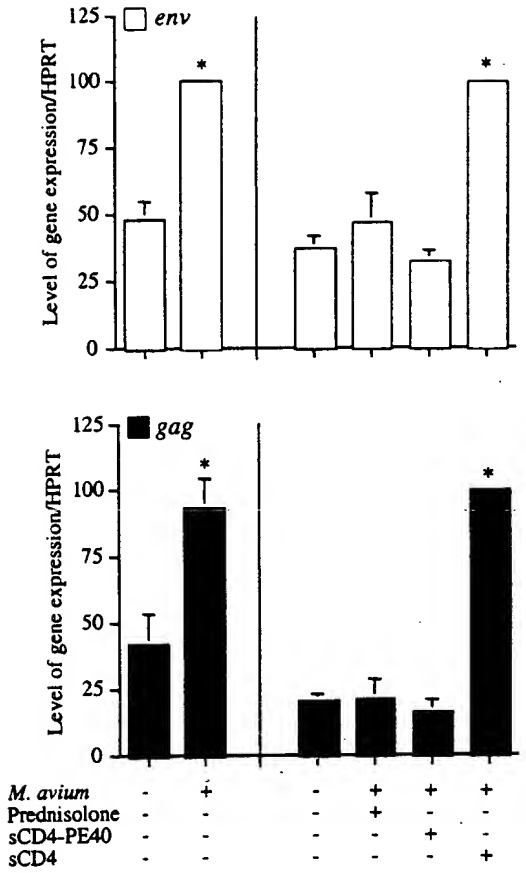


Figure 5. Effects of prednisolone or envelope glycoprotein *(env)*–targeted toxin (soluble CD4 [sCD4]–PE40) administration on *Mycobacterium avium*–induced human immunodeficiency virus type 1 mRNA expression in vivo. mRNA samples were prepared from spleen cells from individual uninfected ($n = 2$) or *M. avium*–infected ($n = 2$) transgenic (Tg) mice *(left)* or from individual infected or control mice treated with prednisolone, sCD4-PE40, or sCD4 *(right)*, as in figures 3 and 4. Real-time reverse transcriptase–polymerase chain reaction for the *env*, *gag*, and *HPRT* genes was done on cDNAs derived from each sample, and amplification was monitored using a LightCycler apparatus (Roche Diagnostics), as described in Materials and Methods. The level of *env* and *gag* gene expression relative to the *HPRT* control was calculated for each sample. Data represent the means calculated from the pooled triplicate determinations done for each mouse in each group, with error bars indicating the SDs. $*P < .01$, compared with uninfected controls.

fects of the *env*-targeted chimeric toxin are specific, because they could be competitively inhibited by unconjugated sCD4. sCD4-PE40 blocked the in vivo elevation in plasma p24 levels induced by *M. avium* infection, without affecting spleen weight or bacteria load. Similar suppressive effects were observed for ex vivo p24 production by spleen cells from *M. avium*–infected Tg mice and the production of infectious virions by the same cell populations, as well as the in situ expression of transcripts for the *env* and *gag* genes. These results for the *env*-targeted chimeric toxin suggest that the line 166 Tg mouse, in addition to being a useful tool for screening inhibitors of immune activation, also can be used effectively to test agents that directly target elements in the virus life cycle.

Although clearly applicable to the examples described here, the Tg mouse model we used has several limitations as an in vivo assay for interventions that target HIV-1 expression. First, because the model only reveals effects on the induction of integrated virus, it cannot be used to assay treatments designed to attack preintegration steps in the HIV-1 life cycle (e.g., inhibitors of RT). Furthermore, because the mouse lacks the necessary surface receptors for the virus, the model is inappropriate for testing inhibitors of HIV-1 fusion or entry (e.g., chemokine-receptor antagonists). An additional limitation that we encountered concerns the ex vivo assay used for measuring in vivo effects of the interventions tested. Although spleen cell cultures revealed a clear-cut blockade of *M. avium*–induced p24 and infectious virion production, these effects were found to be short-lived in vitro, disappearing after 48 h of incubation in the case of either prednisolone or sCD4-PE40 treatment (data not shown). We interpret this ex vivo loss in activity as a re-

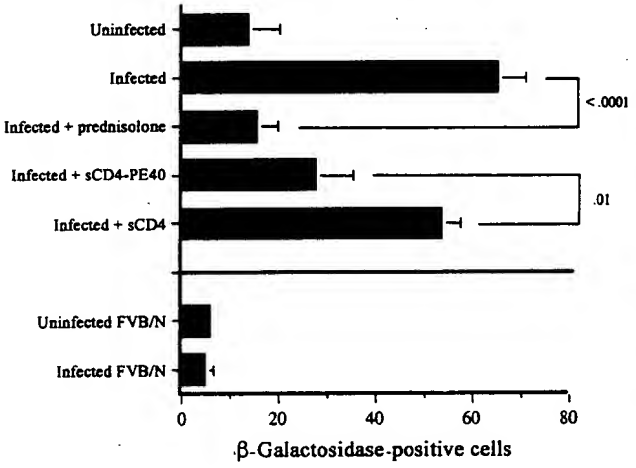


Figure 6. Effects of in vivo inhibitor administration on *Mycobacterium avium*–induced human immunodeficiency virus (HIV) type 1 expression, as measured by the ex vivo production of infectious virions. *M. avium*–infected and uninfected transgenic (Tg) mice were treated with the indicated drugs, as in figures 3 and 4. Mice were killed at 11 days after infection, and spleen cells were cultured for 24 h. Supernatants then were analyzed for infectious HIV-1 virions by using the multinuclear activation of a galactosidase indicator (MAGI) cell assay, in which virus-infected cells are stimulated to express β-galactosidase. Supernatants of spleen cells from *M. avium*–infected and uninfected non-Tg FVB/N mice were analyzed as background controls. Data represent the means for each group of mice: Tg, infected ($n = 4$); Tg, infected, prednisolone treated ($n = 4$); Tg, infected, envelope glycoprotein–targeted toxin (soluble CD4 [sCD4]–PE40) treated ($n = 3$); Tg, infected, sCD4 treated ($n = 4$); Tg, uninfected ($n = 2$); non-Tg, infected ($n = 2$); and non-Tg, uninfected ($n = 2$). Error bars indicate the SDs. The results shown are representative of 2 experiments performed. Nos. on the right are *P* values.

covery of virus expression after the removal of the cells from the suppressive agent during the transition to in vitro culture. In the case of sCD4-PE40, this recovery may reflect the appearance of new *env*-expressing cells during extended culture. Regardless, since the expression of viral mRNA in freshly explanted spleen tissue also is reduced dramatically as a consequence of either sCD4-PE40 or prednisolone treatment, the ex vivo p24 assay appears to faithfully reflect the effects of these interventions on viral induction in vivo.

A further limitation of the line 166 Tg mouse model may actually represent a unique advantage. For reasons that are not yet clear, viral expression in the line 166 Tg mouse (as opposed to another, recently described Tg mouse line that incorporates full-length HIV-1 DNA [26]) cannot be induced from T lymphocytes, even by polyclonal stimulation; instead, viral expression appears to originate primarily from antigen-presenting cells [37–39]. Therefore, interventions that target T cell–specific pathways of HIV-1 induction cannot be tested using these animals. Nevertheless, given the growing interest in non–T lymphocyte reservoirs of HIV-1 infection [40], this restriction may be a useful aspect of the line 166 Tg mouse model. Thus, by offering the ability to rapidly test candidate inhibitors for their activity both in vitro and in vivo, the model may provide a powerful and cost-effective screening tool for the identification of novel interventions for blocking HIV-1 expression from these non–T cell sources.

Acknowledgments

We thank Malcolm Martin, Eric Freed, and Akira Ono (Laboratory of Molecular Microbiology, National Institute for Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH], Bethesda, MD) for their helpful suggestions throughout the course of this project; Claire Chougnet and Gene Shearer (Experimental Immunology Branch, National Cancer Institute [NCI], NIH) and Ettore Appella (Laboratory of Cell Biology, NCI, NIH) for critically reviewing our manuscript; and Brian Vaillant (Schistosomiasis Pathology Unit, Immunobiology Section, Laboratory of Parasitic Diseases, NIAID, NIH) for his advice on the real-time reverse transcriptase–polymerase chain reaction measurements.

References

1. Chun TW, Fauci AS. Latent reservoirs of HIV: obstacles to the eradication of virus. Proc Natl Acad Sci USA 1999;96:10958–61.
2. Ho DD. Toward HIV eradication or remission: the tasks ahead. Science 1998;280:1866–7.
3. Pierson T, McArthur J, Siliciano RF. Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. Annu Rev Immunol 2000;18:665–708.
4. De Haas CJ, de Vos NM, Visser MR, Snippe H, Verhoef J. Monocytes modulate enhancement of HIV-1 replication by *Mycobacterium tuberculosis*. Clin Exp Immunol 1998;111:286–92.
5. Goletti D, Weissman D, Jackson RW, et al. Effect of *Mycobacterium tuberculosis* on HIV replication: role of immune activation. J Immunol 1996; 157:1271–8.
6. Goletti D, Weissman D, Jackson RW, Collins F, Kinter A, Fauci AS. The in vitro induction of human immunodeficiency virus (HIV) replication in purified protein derivative–positive HIV-infected persons by recall antigen response to *Mycobacterium tuberculosis* is the result of a balance of the effects of endogenous interleukin-2 and proinflammatory and antiinflammatory cytokines. J Infect Dis 1998;177:1332–8.
7. Moriuchi H, Moriuchi M, Mizell SB, Ehler LA, Fauci AS. In vitro reactivation of human immunodeficiency virus 1 from latently infected, resting $CD4^+$ T cells after bacterial stimulation. J Infect Dis 2000;181:2041–4.
8. Wahl SM, Greenwell-Wild T, Peng G, et al. *Mycobacterium avium* complex augments macrophage HIV-1 production and increases CCR5 expression. Proc Natl Acad Sci USA 1998;95:12574–9.
9. Brichacek B, Swindells S, Janoff EN, Pirruccello S, Stevenson M. Increased plasma human immunodeficiency virus type 1 burden following antigenic challenge with pneumococcal vaccine. J Infect Dis 1996;174:1191–9.
10. Stanley S, Ostrowski MA, Justement JS, et al. Effect of immunization with a common recall antigen on viral expression in patients infected with human immunodeficiency virus type 1. N Engl J Med 1996;334:1222–30.
11. Staprans SI, Hamilton BL, Follansbee SE, et al. Activation of virus replication after vaccination of HIV-1–infected individuals. J Exp Med 1995;182:1727–37.
12. Andrieu JM, Lu W, Levy R. Sustained increases in CD4 cell counts in asymptomatic human immunodeficiency virus type 1–seropositive patients treated with prednisolone for 1 year. J Infect Dis 1995;171:523–30.
13. Kurata S, Yamamoto N. Glucocorticoid can reduce the transcriptional activation of HIV-1 promoter through the reduction of active NF-κB. J Cell Biochem 1999;76:13–9.
14. Russo FO, Patel PC, Ventura AM, Pereira CA. HIV-1 long terminal repeat modulation by glucocorticoids in monocytic and lymphocytic cell lines. Virus Res 1999;64:87–94.
15. La Maestra L, Zaninoni A, Marriott JB, Lazzarin A, Dalgleish AG, Barcellini W. The thalidomide analogue CC-3052 inhibits HIV-1 and tumour necrosis factor–α (TNF-α) expression in acutely and chronically infected cells in vitro. Clin Exp Immunol 2000;119:123–9.
16. Moreira AL, Corral LG, Ye W, et al. Thalidomide and thalidomide analogs reduce HIV type 1 replication in human macrophages in vitro. AIDS Res Hum Retroviruses 1997;13:857–63.
17. Maciaszek JW, Coniglio SJ, Talmage DA, Viglianti GA. Retinoid-induced repression of human immunodeficiency virus type 1 core promoter activity inhibits virus replication. J Virol 1998;72:5862–9.
18. Towers G, Harris J, Lang G, Collins MK, Latchman DS. Retinoic acid inhibits both the basal activity and phorbol ester–mediated activation of the HIV long terminal repeat promoter. AIDS 1995;9:129–36.
19. Mace K, Gazzolo L. Interferon-regulated viral replication in chronically HIV-1–infected promonocytic U937 cells. Res Virol 1991;142:213–20.
20. Montaner LJ, Bailer RT, Gordon S. IL-13 acts on macrophages to block the completion of reverse transcription, inhibit virus production, and reduce virus infectivity. J Leukoc Biol 1997;62:126–32.
21. Pinto LA, Blazevic V, Patterson BK, Mac Trubey C, Dolan MJ, Shearer GM. Inhibition of human immunodeficiency virus type 1 replication prior to reverse transcription by influenza virus stimulation. J Virol 2000;74: 4505–11.
22. Schnittman SM, Vogel S, Baseler M, Lane HC, Davey RT, Jr. A phase I study of interferon-α2b in combination with interleukin-2 in patients with human immunodeficiency virus infection. J Infect Dis 1994;169:981–9.
23. Kutza J, Crim L, Feldman S, et al. Macrophage colony-stimulating factor antagonists inhibit replication of HIV-1 in human macrophages. J Immunol 2000;164:4955–60.
24. Poli G, Kinter AL, Fauci AS. Interleukin 1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin 6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin 1 receptor antagonist. Proc Natl Acad Sci USA 1994;91:108–12.
25. Walker RE, Spooner KM, Kelly G, et al. Inhibition of immunoreactive tumor

necrosis factor–α by a chimeric antibody in patients infected with human immunodeficiency virus type 1. J Infect Dis 1996;174:63–8.

26. Browning PJ, Wang EJ, Pettoello-Mantovani M, et al. Mice transgenic for monocyte-tropic HIV type 1 produce infectious virus and display plasma viremia: a new in vivo system for studying the postintegration phase of HIV replication. AIDS Res Hum Retroviruses 2000;16:481–92.
27. De SK, Wohlenberg CR, Marinos NJ, Doodnauth D, Bryant JL, Notkins AL. Human chorionic gonadotropin hormone prevents wasting syndrome and death in HIV-1 transgenic mice. J Clin Invest 1997;99:1484–91.
28. Dickie P. Nef modulation of HIV type 1 gene expression and cytopathicity in tissues of HIV transgenic mice. AIDS Res Hum Retroviruses 2000;16: 777–90.
29. Dickie P, Felser J, Eckhaus M, et al. HIV-associated nephropathy in transgenic mice expressing HIV-1 genes. Virology 1991;185:109–19.
30. Dickie P, Gazzinelli R, Chang LJ. Models of HIV type 1 proviral gene expression in wild-type HIV and MLV/HIV transgenic mice. AIDS Res Hum Retroviruses 1996;12:1103–16.
31. Dickie P, Mounts P, Purcell D, et al. Myopathy and spontaneous *Pasteurella pneumotropica*–induced abscess formation in an HIV-1 transgenic mouse model. J Acquir Immune Defic Syndr Hum Retrovirol 1996;13:101–16.
32. Hanna Z, Kay DG, Cool M, Jothy S, Rebai N, Jolicoeur P. Transgenic mice expressing human immunodeficiency virus type 1 in immune cells develop a severe AIDS-like disease. J Virol 1998;72:121–32.
33. Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur P. Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. Cell 1998;95:163–75.
34. Santoro TJ, Bryant JL, Pellicoro J, et al. Growth failure and AIDS-like cachexia syndrome in HIV-1 transgenic mice. Virology 1994;201:147–51.
35. Tinkle BT, Ueda H, Ngo L, et al. Transgenic dissection of HIV genes involved in lymphoid depletion. J Clin Invest 1997;100:32–9.
36. Gazzinelli RT, Sher A, Cheever A, Gerstberger S, Martin MA, Dickie P. Infection of human immunodeficiency virus 1 transgenic mice with *Toxoplasma gondii* stimulates proviral transcription in macrophages in vivo. J Exp Med 1996;183:1645–55.
37. Doherty TM, Chougnet C, Schito M, et al. Infection of HIV-1 transgenic mice with *Mycobacterium avium* induces the expression of infectious virus selectively from a Mac-1–positive host cell population. J Immunol 1999; 163:1506–15.
38. Chougnet C, Freitag C, Schito M, Thomas EK, Sher A, Shearer GM. In vivo CD40-CD154 (CD40 ligand) interaction induces integrated HIV expression by APC in an HIV-1–transgenic mouse model. J Immunol 2000; 166:3210–3217.
39. Freitag C, Chougnet C, Schito M, et al. Malaria infection induces virus expression in human immunodeficiency virus transgenic mice by CD4 T cell–dependent immune activation. J Infect Dis 2001;183:1260–8.
40. Martin JC, Bandres JC. Cells of the monocyte-macrophage lineage and pathogenesis of HIV-1 infection. J Acquir Immune Defic Syndr 1999;22: 413–29.
41. Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Colch) 1998;94:557–72.
42. Chaudhary VK, Mizukami T, Fuerst TR, et al. Selective killing of HIV-infected cells by recombinant human CD4-*Pseudomonas* exotoxin hybrid protein. Nature 1988;335:369–72.
43. Berger EA, Moss B, Pastan I. Reconsidering targeted toxins to eliminate HIV infection: you gotta have HAART. Proc Natl Acad Sci USA 1998;95: 11511–3.
44. Goldstein H, Pettoello-Mantovani M, Bera TK, Pastan IH, Berger EA. Chimeric toxins targeted to the human immunodeficiency virus type 1 envelope glycoprotein augment the in vivo activity of combination antiretroviral therapy in thy/liv-SCID-Hu mice. J Infect Dis 2000;181:921–6.
45. Hurtenbach U, Boggemeyer E, Stehle T, Museteanu C, Del Pozo E, Simon MM. Prednisolone reduces experimental arthritis, and inflammatory tissue destruction in SCID mice infected with *Borrelia burgdorferi*. Int J Immunopharmacol 1996;18:281–8.
46. Doherty TM, Sher A. IL-12 promotes drug-induced clearance of *Mycobacterium avium* infection in mice. J Immunol 1998;160:5428–35.
47. Kimpton J, Emerman M. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J Virol 1992;66: 2232–9.
48. Ashorn P, Englund G, Martin MA, Moss B, Berger EA. Anti-HIV activity of CD4-*Pseudomonas* exotoxin on infected primary human lymphocytes and monocyte/macrophages. J Infect Dis 1991;163:703–9.

An Anti-CD45RO Immunotoxin Kills Latently Infected Human Immunodeficiency Virus (HIV) CD4 T Cells in the Blood of HIV-Positive Persons

Jesús Saavedra-Lozano,[1,2] Cynthia McCoig,[1,2] Jinbo Xu,[1] Yanying Cao,[1] Philip Keiser,[4] Victor Ghetie,[1,3] Robert F. Siliciano,[5] Janet D. Siliciano,[5] Louis J. Picker,[1,6] Octavio Ramilo,[1,2,3,a] and Ellen S. Vitetta[1,3,a]

[1]Cancer Immunobiology Center and Departments of [2]Pediatrics, [3]Microbiology, and [4]Internal Medicine, University of Texas Southwestern Medical Center, Dallas; [5]Johns Hopkins University, Baltimore, Maryland; [6]Veterans Affairs Medical Center, Portland, Oregon

Highly active antiretroviral therapy has decreased the morbidity and mortality of human immunodeficiency virus (HIV) infection, but latently infected cells remain for prolonged periods. $CD4^+$ $CD45RO^+$ T cells are a major latent virus reservoir in HIV-infected persons. Replication-competent, latently HIV-infected T cells can be generated in vitro by infecting peripheral blood mononuclear cells with HIV and then eliminating the HIV-producing cells with an anti-CD25 immunotoxin (IT). The $CD25^-$ latently infected cells then can be eliminated with an anti-CD45RO IT. This study determined whether this IT also could kill latently infected CD4 T cells from HIV-infected persons with or without detectable plasma viremia. The results show that ex vivo treatment of cells from HIV-positive persons by anti-CD45RO IT reduces the frequency of both productively and latently infected cells. In contrast, $CD4^+$ $CD45RA^+$ naive T cells and a proportion of $CD4^+$ $CD45RO^{lo}$ memory T cells are spared.

Current treatment guidelines for human immunodeficiency virus (HIV) infection recommend the use of highly active antiretroviral therapy (HAART) to maximally reduce the levels of circulating virus [1]. Even when treatment is successful and virus levels in plasma fall below the level of detection, long-lived latently infected cells remain [2–7], and the withdrawal of HAART almost invariably leads to a rebound in viremia [8]. Although there are several potential virus reservoirs, including macrophages [9], microglial cells in the central nervous system [9], seminal fluid cells (which may include macrophages and/or T cells) [9–11], thymocytes [12], and resting CD4 T memory cells [9], long-term persistence of HIV has been best documented in $CD45RO^+$ T cells [2, 3, 9, 13, 14]. Previous studies showed a biphasic decay in the frequency of these latently infected resting CD4 T cells after the initiation of HAART [3, 15–18]. The more rapid initial phase may reflect the decay of cells with linear nonintegrated HIV-1 DNA. The remaining pool of latently infected cells is extremely stable and is most likely due to the long-term survival of cells in the postintegration phase of latency [3, 5, 9, 19].

When considering the side effects and cost of long-term HAART, it is important to develop new strategies to reduce or eliminate latent virus reservoirs or to prevent them from becoming activated. One strategy is to stimulate the latent T cells with interleukin (IL)–2 in the presence of HAART, to exhaust the reservoir [19–23]. Another strategy is to intensify the antiretroviral therapy, to further decrease viral replication so that the reservoir cannot be sustained [9, 16]. In addition, cyclosporine A, an immunosuppressive drug with some anti-HIV activity, has been used to prevent these resting cells from becoming activated [24–27], in some cases with significant and sustained increases in numbers of CD4 T cells [26]. Recently, investigators evaluated cyclophosphamide in combination with HAART but found no effect on HIV DNA [28]. A variety of vaccine approaches currently under investigation [29–31] might also decrease latent reservoirs, but this remains to be tested.

Our work has focused on the development of immunotoxins (ITs) to kill $CD25^-$ resting latently infected T cells. In previously published studies, normal peripheral blood mononuclear cells (PBMC) first were infected with HIV in vitro and then were treated with an anti-CD25 IT to eliminate $CD25^+$ activated T cells producing virus [32–34]. The resting $CD25^-$ cells remaining after treatment did not produce HIV unless they were activated [33]; these cells harbored nonintegrated, incomplete reverse-transcribed DNA transcripts, but lacked full-length (FL), reverse-transcribed viral DNA [35]. By using this in vitro model of prein-

Received 20 July 2001; revised 4 October 2001; electronically published 8 January 2002.

Presented in part: 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, September 2000 (abstract 1165).

The study was approved by the University of Texas Southwestern Medical Center at Dallas institutional review board. Informed consent was obtained from each subject before blood samples were obtained.

Financial support: National Institutes of Health (AI-43222); State of Texas Higher Education Coordinating Board (010019-0100).

[a] O.R. and E.S.V. codirected this study.

Reprints or correspondence: Dr. Ellen S. Vitetta, University of Texas Southwestern Medical Center, Cancer Immunobiology Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8576 (Ellen.Vitetta@UTSouthwestern.edu).

The Journal of Infectious Diseases 2002;185:306–14

0022-1899/2002/18503-0005$02.00

tegration latency, we showed that the anti-CD45RO IT, UCHL-1 deglycosylated ricin A chain (dgRTA), virtually eliminated $CD25^-$ latently infected cells [34].

In the present study, we determined whether this anti-CD45RO IT would kill latently infected cells of HIV-positive persons with or without detectable viremia. We also analyzed the in vitro effect of this IT on T cells from these subjects.

Methods

Study design. The objective of this study was to determine whether an IT against CD45RO could reduce the frequency of CD4 latent, replication-competent, HIV-infected T cells from HIV-positive subjects with or without detectable viremia. This was accomplished by using limiting dilution cultures in which triplicates of 4 different concentrations of purified CD4 T cells were incubated for 6 days with complete medium (CM), an anti-CD25 IT, an anti-CD45RO IT, or a combination of both ITs. The remaining cells then were cocultured with phytohemagglutinin (PHA)–activated PBMC to recover replication-competent virus, as determined by p24 production. The frequency of HIV-infected cells was determined by use of multiple regression analysis, as described elsewhere [36–38].

ITs. ITs were prepared by coupling monoclonal antibodies (MAbs) to dgRTA, as described elsewhere [39]. The following MAbs were used: UCHL-1, a murine IgG2a directed against human CD45RO [40], and RFT5, a murine IgG1 directed against human CD25 [41]. Previous studies using irrelevant ITs showed that they do not nonspecifically kill cells lacking the target antigen [34].

HIV-positive patients. HIV-infected patients with detectable viremia were recruited from the Amelia Court Clinic (University of Texas Southwestern Medical Center, Dallas). All subjects had detectable HIV plasma viremia and CD4 cell counts >200 cells/mm^3. Subjects were enrolled regardless of past or present treatments. The 3 patients with plasma viremia below the limit of detection (<50 copies HIV RNA/mL) who were receiving HAART were recruited from the Moore Clinic (Johns Hopkins University, Baltimore).

Limiting dilution cultures and ex vivo treatment of cells with ITs. For the samples prepared at the University of Texas, fresh PBMC were obtained by centrifugation of heparinized blood over ficoll-paque. CD4 T cells then were isolated by negative selection, using CD4 T cell isolation kits (Miltenyi Biotec). Cells were cultured in limiting dilution in triplicate wells in 24-well plates with 4 different cell concentrations (range, 5×10^4–5×10^6 cells/well). Cells were cultured in either CM (RPMI 1640, 15% heat-inactivated fetal calf serum, L-glutamine, 10% IL-2, and antibiotics) or CM plus ITs at a concentration of 10 n*M* each. Cells were treated with either the anti-CD25 IT, the anti-CD45RO IT, or the combination of the two. After 6 days in culture, cells were washed twice with RPMI 1640 and were cocultured for 6 days with 10^6 PHA-activated allogeneic PBMC/well. These PBMC were obtained from HIV-negative donors and were cultured with PHA for 3 days before their addition to the cultures. On day 12, cell supernatants were harvested, and p24 levels were determined. Cell frequencies were determined by using a multiple regression equation, which correlates the cell concentration with the percentage of wells lacking detectable levels of p24 production [36–38]. This method is based on the hypothesis that the number of functional cells per volume of sample follows a Poisson distribution and that, at a cell concentration at which 66% of the wells are negative, there is 1 functional (i.e., HIV-producing) cell per well [37]. A p24 concentration of ≥30 pg/mL was considered to be positive for virus production. In some experiments, cells were harvested 6 days after IT treatment and before coculture for flow cytometric analysis. For the 3 patients studied at Johns Hopkins, CD4 cells were obtained by magnetic bead depletion of B cells, monocytes, CD8 cells, and NK cells, as described elsewhere [4, 13, 42].

Resting CD4 cells were isolated by removing activated CD4 T cells, using MAbs against CD25, CD69, and HLA-DR. Resting cells were treated with IT for 6 days, with fresh IT added on day 3. Cells then were washed and counted. The frequency of latently infected cells was determined using a previously described limiting dilution culture assay [2–4]. Input cell concentrations were normalized on the basis of the number of cells in control wells after 6 days of incubation.

Statistical analysis. Regression lines were calculated and plotted by using SigmaPlot 2000 software (SPSS Science).

Flow cytometric analysis. Six-parameter flow cytometric analysis was performed on a 4-color FACSCalibur flow cytometer (Becton Dickinson). The criteria for defining and quantifying memory and naive T cells have been described elsewhere [43]. Naive cells are $CD95^{lo}$, $CD27^{hi}$, $CD45RO^-$, and $CD45RA^+$, and memory cells are $CD95^{hi}$, $CD27^{heterogeneous}$, $CD45RO^+$, and $CD45RA^{lo/-}$. Small numbers of $CD4^+$ memory T cells and up to 50%–80% of CD8 memory T cells are $CD45RO^{-/lo}$, $CD45RA^+$.

p24 assays. The production of p24 antigen in cell-free supernatants was measured using a commercial ELISA kit (NEN Life Science Products). Values were expressed as picograms per milliliter. Wells were considered to be positive when p24 levels were ≥30 pg/mL.

Polymerase chain reaction (PCR). In each experiment, ~10^6 CD4 T cells were used for PCR, as described elsewhere [33, 35]. As an internal standard for HIV DNA, we used 8E5 cells from the National Institutes of Health AIDS repository; 8E5 cells contain a single integrated copy of proviral HIV DNA per cell [44]. The viral genome was amplified by primers corresponding to RU5 and FL HIV DNA, as described elsewhere [33, 35]. We used RU5 to identify early HIV-1 DNA fragments. We also used FL to demonstrate the formation of nearly completely synthesized HIV-1 DNA, since FL represents the last region of the minus strand of viral DNA that is synthesized. The amplified FL region of the HIV-1 genome corresponds to a 161-bp fragment spanning residues 43–203 [33, 35]. Normal PBMC from seronegative donors were used as negative controls. This assay can routinely detect 1 copy of HIV proviral DNA/10^6 cells when RU5 is analyzed and 10 copies/10^6 cells when FL is analyzed.

Results

Patients with detectable viremia. Blood samples from 13 HIV-infected persons with detectable plasma viremia who were undergoing a variety of therapies were obtained from the University of Texas (table 1). CD4 cell counts were 193–1067 cells/μL

Table 1. Antiretroviral therapy, plasma virus load, and CD4 T cell count of each subject at study enrollment.

Group, patient	Treatment	Virus load, HIV RNA copies/mL	CD4 cell count, cells/μL
Detectable viremia			
1	Naive	19,547	327
2	Naive	404,835	251
3	Naive	18,561	512
4	None	59,910	688
5	None	39,000	547
6	None	7921	460
7	RemmuneÒ	1234	524
8	3TC/ddI/Nfv	103,521	419
9	ddI/d4T/Nfv	4508	754
10	None	2626	482
11	Idv/Zdv/3TC	3963	635
12	ddI/d4T/Nfv	120,020	193
13	Nfv/Zdv/3TC	73,754	1067
Viremia below level of quantitation			
14	Zdv/3TC/Idv	<50	844
15	Zdv/3TC/Abc/Efv	<50	1396
16	d4T/3TC/Nfv	<50	989

NOTE. 3TC, lamivudine; Abc, abacavir; d4T, stavudine; ddI, didanosine; Efv, efavirenz; HIV, human immunodeficiency virus; Idv, indinavir; Nfv, nelfinavir; Zdv, zidovudine.

(median, 512 cells/μL). Levels of HIV RNA in plasma were 2626–404,835 copies/mL (median, 19,547 copies/mL).

Patients with viremia below the levels of detection. Blood samples from 3 patients receiving HAART who had prolonged suppression of plasma viremia (<50 HIV RNA copies/mL) for 47–56 months were obtained by apheresis at Johns Hopkins (table 1). Previous studies documented the persistence of stable reservoirs of latent HIV in resting CD4 T cells in these same patients [2–4, 13].

Flow cytometric analysis of IT-treated CD4 T cells. To characterize the effect of treatment with the ITs on the different lymphocyte subpopulations, samples from 3 additional persons were analyzed after culture with CM or ITs and before activation with PHA-stimulated PBMC. Treated cells were analyzed for the expression of CD25, CD45RA, and CD45RO and for memory and naive phenotypes [34] (figure 1 and table 2). Both the anti-CD25 and anti-CD45RO ITs eliminated their target cells in a highly effective and specific fashion (table 2). Flow cytometric analysis demonstrated that 5.6%–7.1% of CM-treated cells (controls) were $CD25^+$. Treatment with the anti-CD25 IT yielded a population of T cells that contained <1% $CD25^+$ cells (table 2) and were, therefore, resting cells. Treatment with the anti-CD45RO IT reduced the percentage of remaining $CD45RO^+$ T cells from 33%–50% to 1.5%–3% (table 2), and surviving cells were $CD45RO^{lo}$. When cells were treated with the combination of the anti-CD25 and anti-CD45RO ITs (table 2), comparable reductions in the numbers of both $CD25^+$ and $CD45RO^+$ T cells were observed. None of these ITs decreased the percentage of cells lacking the target antigen. Hence, the percentage of $CD45RA^+$ T cells was 47%–60% in CM-treated cells and 34%–71% in cells treated with the different ITs (table 2). Therefore, as shown elsewhere by using cells infected with HIV in vitro, both the anti-CD25 and anti-CD45RO ITs are effective and specific [34].

When CD4 memory T cells were analyzed in control CM-treated cells, there were 53%–67% memory cells (both $CD45RO^{hi}$ and $CD45RO^{lo}$; table 2). After treatment with the anti-CD45RO IT, 6%–14% $CD45RO^{lo}$ memory cells remained. A similar percentage of $CD45RO^{lo}$ memory cells (4%–19%) remained after treatment with the combination of the anti-CD25 plus the anti-CD45O ITs (table 2). The surviving $CD45RO^{lo}$ memory cells were not productively infected, as determined by the lack of p24 production after activation (see below).

HIV latently infected cells from persons with detectable plasma viremia are eliminated ex vivo with either the anti-CD45RO IT alone or in combination with the anti-CD25 IT. CD4 T

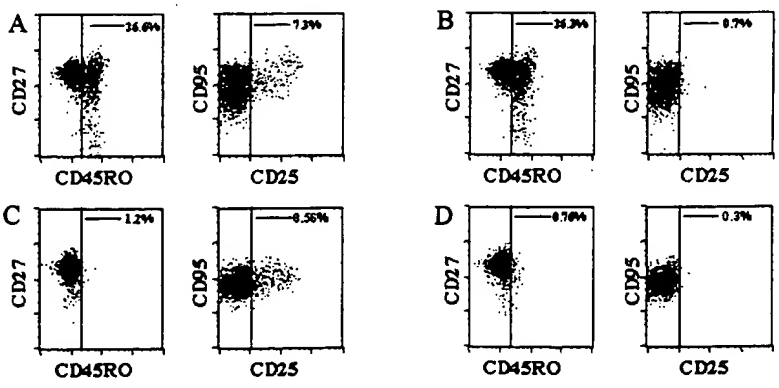


Figure 1. Flow cytometric analysis of CD4 T cells 6 days after treatment with different immunotoxins (ITs) in 1 patient. *Left panels*, $CD45RO^+$ cell percentages: 36.6% after incubation with control medium (CM; *A*), 36.3% after treatment with anti-CD25 IT *(B)*, 1.2% after treatment with anti-CD45RO IT *(C)*, and 0.76% after treatment with both ITs *(D)*. *Right panels*, $CD25^+$ cell percentages: 7.3% after culture with CM *(A)*, 0.7% after culture with anti-CD25 IT *(B)*, 8.56% after culture with anti-CD45RO IT *(C)*, and 0.3% after culture with both ITs *(D)*.

Table 2. Summary of flow cytometric analyses of cells from 3 subjects after incubation with different immunotoxins (ITs).

IT	Phenotype, %				
	Memory[a]	Naive[b]	CD45RO$^+$	CD45RA$^+$	CD25$^+$
Control medium	60 (53–67)	40 (33–47)	41 (33–50)	52 (47–60)	6.3 (5.6–7.1)
Anti-CD25	51 (41–60)	49 (39–59)	40 (33–45)	42 (34–55)	0.4 (0.4–0.5)
Anti-CD45RO	9.5 (6–14)	90 (86–94)	2 (1.5–3.0)	59 (56–61)	12 (8.9–15.2)
Anti-CD25 plus anti-CD45RO	12 (4–19)	88 (81–95)	1.5 (1.1–2.0)	64 (53–71)	0.8 (0.7–0.9)

NOTE. Data are mean (range).

[a] Memory cells are defined as CD95^{hi}, CD45RO$^+$, CD27^{heterogeneous}, CD45RA$^{lo/-}$.

[b] Naive cells are defined as CD95^{lo}, CD27^{hi}, CD45RO$^-$, and CD45RA$^+$.

cells were isolated and cultured in limiting dilution for 6 days with either CM, the anti-CD25 IT, the anti-CD45RO IT, or a combination of both. Cells then were cocultured with PHA-activated allogeneic PBMC to activate the remaining viable cells, to detect productively infected, replication-competent, cells by measuring p24 concentrations in cell supernatants.

We used multiple regression analysis to calculate the frequencies of HIV replication–competent CD4 cells [36–38] (figure 2). The regression coefficients (r^2) always were >.8. Because of the range of concentrations of cells that were used in the limiting dilution assays, the frequency detected was 0.6–25 HIV-infected cells/10^6 CD4 T cells.

Cells obtained from patients 2, 4, 6, 8, 9, and 13 were treated with the anti-CD25 IT and then were cocultured in limiting dilution with PHA-activated cells. Since the anti-CD25 IT eliminates the cells actively producing HIV, coculturing the CD25$^-$ CD4$^+$ T cells with PHA-activated PBMC allowed us to determine the frequency of latently infected cells (which can subsequently produce HIV after activation) [32, 33]. As shown in table 3, p24 was detected in the cultures of all 6 samples evaluated, and there were 5 to >20 latently infected cells/10^6 CD4 T cells (table 3).

When we analyzed the cells cultured with anti-CD45RO IT alone or in combination with the anti-CD25 IT, we could not detect p24 in any of the microwells containing cells treated with either the anti-CD45RO IT (8 patients; table 3), or the anti-CD25 plus CD45RO ITs (10 patients; table 3). Therefore, the frequency of HIV-infected cells (both productively and latently infected) in cultures treated with anti-CD45RO IT was below the limit of detection of our assay (<1 HIV-infected cell/10^6 CD4 T cells; table 3). These results indicate that, after treatment with the anti-CD45RO IT, the number of HIV-infected cells was reduced by a minimum of 3–20-fold.

FL HIV DNA provirus from CD4 T cells could not be detected after treatment with anti-CD45RO IT and subsequent activation of surviving cells. PCR was performed on CD4 T cells obtained from 5 subjects after 6 days in culture with or without ITs, followed by 6 days of coculture with PHA-activated allogenic PBMC. After coculture, DNA was extracted from the cells, and RU5 and FL fragments were amplified by PCR. RU5 and FL fragments were identified in activated CD4 T cells that had been treated with the anti-CD25 IT (2 samples; figure 3 and table 4). p24 production also was detected in the supernatants of these samples. In contrast, RU5 but not FL, DNA could be detected in activated CD4 T cells that had been treated with the anti-CD45RO IT either alone (4 samples) or in combination with the anti-CD25 IT (5 samples). Compared with cells treated with CM or the anti-CD25 IT (and then activated), p24 was never detected in activated cells that were treated previously with the anti-CD45RO IT. Although the PCR used was not quantitative, the presence of FL DNA correlated with the detection of p24 in the samples analyzed, and FL DNA could not be detected in cells treated with anti-CD45RO IT.

The anti-CD45RO IT significantly reduced the frequency of latently infected cells obtained from patients with plasma viremia below the limit of detection who were receiving HAART. Patients with plasma viremia below the limit of detection who had been receiving HAART for long periods (patients 14–16) and were documented previously to have cells in the postintegration phase of latency [2, 13] were studied to assess the effects of ITs on these cells (table 3). Highly purified resting CD4 T cells were isolated from these patients, as described elsewhere [13], and the cells were cultured for 6 days with either CM or with the anti-CD45RO IT. The frequency of highly purified CD4 HIV latently infected cells determined by a limiting dilution culture assay [2] was 0.1–6.0/10^6 resting CD4 T cells in the blood of these patients. Treatment with anti-CD45RO IT significantly reduced the frequency of these latently infected cells. In patient 14, latently infected CD4 T cells were reduced by 95% (i.e., from 6 to 0.3/10^6 resting CD4 T cells). Likewise, there was an 81% reduction in latently infected cells in patient 15 (from 2.7 to 0.5/10^6 CD4 cells). Patient 16, who had few latent cells to begin with, showed a 60% reduction (from 0.1 to 0.04/10^6 cells). These results indicate that, after treatment with anti-CD45RO IT, the number of HIV latently infected CD4 cells obtained from persons with no detectable plasma viremia and integrated viral genome was decreased, but this reduction varied among the 3 patients tested (table 3). Taken together with the data from patients 1–13, these results suggest that the anti-CD45RO IT kills not only the productively infected but also many of the latently infected CD4 T cells harboring both integrated and nonintegrated HIV DNA.

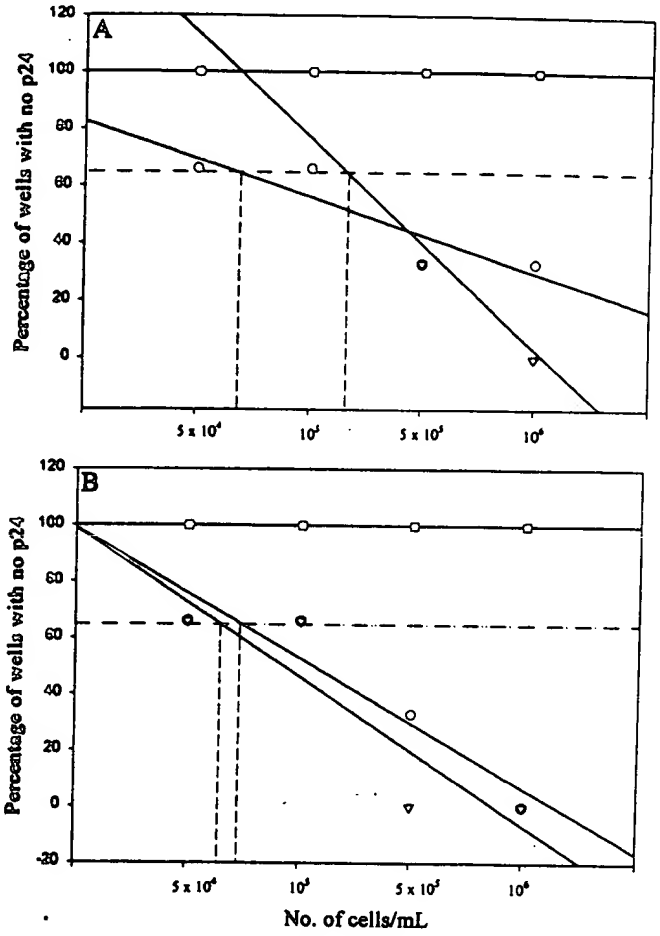


Figure 2. Regression curves from limiting dilution assays on CD4 cells of 2 patients. By multiple regression analysis, frequencies of human immunodeficiency virus (HIV) type 1–infected cells/10^6 CD4 T cells in each subject were determined. *A*, Patient 9 had plasma viremia of 4508 HIV RNA copies/mL and a CD4 cell count of 754 cells/μL. Nos. of HIV-1–infected cells after treatment with complete medium (CM; ○) or anti-CD25 immunotoxin (IT; ▽) were 14 and 5/10^6 $CD4^+$ T cells, respectively. After treatment with a combination of anti-CD25 and anti-CD45RO ITs (□), this frequency decreased to <1/10^6 cells (r^2 = .80 for CM, 0.90 for CD25, and 1 for CD25/45RO). *B*, Patient 2 had plasma viremia of 404,835 HIV RNA copies/mL and CD4 cell count of 251 cells/μL. Frequencies of HIV-1–infected cells after treatment with CM (○), anti-CD25 IT (▽), or a combination of anti-CD25 and anti-CD45RO ITs (□) were 14, 15, and <1 cells/10^6 CD4 T cells, respectively (r^2 = .89 for CM, .90 for CD25, and 1 for CD25/45RO).

Discussion

HIV latency remains a significant obstacle in the management f HIV infection [2, 7–9, 14]. Although there are several reserv irs for the virus, a maj r one is the reservoir of latently infected resting CD4 T cells in the lymphoid tissues and blood [5, 9, 12, 14, 16, 20, 45–47]. To what extent elimination of this T cell reservoir will affect virus rebound after the withdrawal of HAART is unknown. To date, few approaches have been aimed at actually killing the cells in the latent reservoir. Several groups have used IL-2 activation to "flush" these cells out in the presence of HAART [19–23, 48] with some encouraging results. When combined with HAART, there have been increases in CD4 T cells, reductions in plasma viremia [20, 22], and, in some cases, difficulty in recovering virus from the remaining resting T cells [20]. However, there are several concerns about the long-term effects of IL-2 on the immune system [19], its potential toxicity [19, 22], and the virus rebound observed in the plasma of HIV-infected persons receiving IL-2 after HAART is withdrawn [48]. Other studies have focused on the use of immunomodulatory agents to prevent activation of these cells [24–26], even though these agents are generally immunosuppressive.

More recently, other investigators evaluated the effect of therapy with cyclophosphamide, a cytotoxic agent, in combination with HAART on the latent reservoir. No significant reductions in HIV DNA in either PBMC or lymphoid tissue were associated with the administration of cyclophosphamide [28]. Finally, there has been some success in treating patients with HAART soon after infection and then withdrawing HAART at a later time [49]. Some of these patients can control residual viral replication (probably derived from the latent virus reservoirs) for at least several months. Stimulation of anti-HIV cytotoxic T lymphocytes with HIV-based vaccines has been suggested as a way to control virus emerging from latently infected cells [16, 29, 49]. This is also a viable and logical strategy, but it is too early to know how effective it will be in eliminating latently infected cells in vivo.

We explored the possibility of killing resting latently infected T cells with ITs. To this end, we previously developed an in vitro model of HIV preintegration latency in which the $CD25^-$ latently infected T cells produced HIV only after activation [32, 33]. These cells have many of the functional hallmarks of latently infected cells from HIV-positive patients; however, we were unable to generate in vitro–infected cells with postintegration latency [35]. By using these in vitro latently infected cells in conjunction with flow cytometry and PCR, we found that the anti-CD45RO IT effectively killed these cells but spared the naive $CD45RA^+$ cells [34]. Furthermore, this IT preserved a significant percentage of $CD45RO^{lo}$ memory T cells, and these cells were functional, as determined by cytokine expression in the absence of virus production after activation [34]. Of importance, the anti-CD45RO IT was equally effective in killing cells harboring both M-tropic or T-tropic virus, which suggests that this IT should not be influenced by viral mutations [34]. On the basis of these results, we concluded that the anti-CD45RO IT was as effective as the anti-CD25 IT in eliminating activated HIV-producing cells but, of more importance, unlike the anti-CD25 IT, it also killed latently infected cells.

In the present study, we determined whether the anti-CD45RO IT could eliminate latently infected CD4 T cells obtained from HIV-positive persons with or without detectable plasma viremia.

Table 3. Effect of treatment with immunotoxins (ITs) on p24 production and frequency of infected cells.

	p24, pg/mL (range)				Infected cells/10^6				
Group	Patient	CM	CD25	CD45RO	CD25/ CD45RO	CM	CD25[b]	CD45RO	CD25/ CD45RO[c]	Anti-CD45RO IT
Detectable viremia										
	1	95	—	0	—	25	—	<1	—	
	2	75,742 (44,000–100,000)	42,254 (10,563–64,800)	—	0	14	15	—	<1	
	3	300	—	0	0	18	—	<1	<1	
	4	2580 (1300–3650)	3060 (2470–3180)	—	0	20	13	—	<1	
	5	158	—	0	—	5	—	<1	—	
	6	31,833 (12,000–57,900)	65,600 (49,000–86,500)	—	0	>20	>20	—	<1	
	7	68.5	—	0	—	1.3	—	<1	—	
	8	136 (34–358)	147 (34–336)	—	0	3	5	—	<1	
	9	138 (41 –366)	425 (75–990)	—	0	14	5	—	<1	
		43.6	—	0	0	<1	—	<1	<1	
	11	1878	—	0	0	4	—	<1	<1	
	12	925	—	0	0	>20	—	<1	<1	
	13	24,764 (3325–53,360)	25,680 (5437–54,071)	0	0	5	5	<0.6	<0.6	
Viremia below level of detection										
	14[d]					6				0.3
	15[d]					2.7				0.5
	16[d]					0.1				0.04

NOTE. Limiting dilution assay can detect 1 human immunodeficiency virus (HIV)–positive cell/10^6 cells. CM, control medium; —, not done.

[a] 10^6 cells/well.

[b] Group corresponds to no. of HIV latently infected CD4 T cells (see text).

[c] Difference between no. of cells in this group compared with anti-CD25 IT-treated group corresponds to no. of HIV latently infected cells eliminated.

[d] Two experiments were done by using cells from patient 14 (1 shown); 1 experiment each was done by using cells from patients 15 and 16.

The latter patients had documented postintegration latency, as determined by an inverse PCR strategy to selectively amplify the integrated form of HIV-1 DNA [2, 13].

Three major findings emerged from our study. First, by using cells from patients with detectable plasma viremia, the anti-CD45RO IT significantly reduced the frequency of both productively and latently infected CD4 T cells but spared the $CD45RA^+$ cells and some of the $CD45RO^{lo}$ memory cells. Second, the surviving cells did not produce FL HIV DNA or p24 after activation, which suggests that this IT kills a significant proportion of CD4 T cells that may represent replication-competent virus reservoirs. Third, when cells from 3 patients with no detectable viremia who were receiving HAART were evaluated, the anti-CD45RO IT also was highly effective in killing the latently infected cells in samples from 2 of the 3 patients (81%–95% reduction in frequency of HIV-producing cells). In the third patient, who had few latent cells, the IT decreased these cells by 60%. Other studies that assessed this particular patient reported a $CD45RA^+$ (or a $CD45RA^{hi}$, $CD45RO^{lo}$) reservoir [42].

This study demonstrates the utility of these ITs in defining populations of cells infected with HIV. In considering this IT for future clinical development, many important issues must be addressed. These include the possible toxicity of the IT on other hematopoietic cells such as neutrophils or macrophages, how treatment will affect the thymus or functional T cell memory, and the toxicities of ITs [50] plus HAART in HIV-infected persons.

We do not yet know whether naive CD8 T cells and some CD4 $CD45RO^{lo}$ memory T cells are spared. There is evidence from other studies [51–53] that a proportion of CD8 memory T cells and naive CD8 T cells lack CD45RO and should also be spared [34]. Although expression of CD45RA and CD45RO has long been assumed to delineate naive versus memory T cells, recent studies have shown clearly that some memory cells, particularly

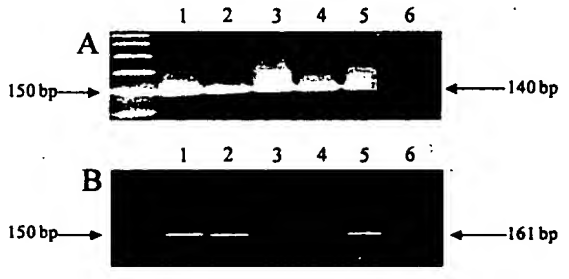


Figure 3. Polymerase chain reaction analysis of human immunodeficiency virus (HIV) proviral DNA from CD4 T cells from HIV-infected persons. *Lanes 1–4*, CD4 cells from HIV-positive subjects; *lane 5*, 8E5 cells; *lane 6*, normal peripheral blood mononuclear cells. *A*, RU5. *B*, Full-length HIV DNA. CD4 cells were treated with complete medium *(lane 1)*, anti-CD25 immunotoxin (IT; *lane 2*), anti-CD45RO IT *(lane 3)*, and both ITs *(lane 4)*.

Table 4. Polymerase chain reaction analysis of human immunodeficiency virus (HIV) proviral DNA from CD4 T cells from HIV-positive persons.

	Control medium			CD25			CD45RO			CD25/CD45RO		
Patient	RU5[a]	FL[b]	p24[c]	RU5[a]	FL[b]	p24[c]	RU5[a]	FL[b]	p24[c]	RU5[a]	FL[b]	p24[c]
3	+	+	+				+	−	−	+	−	−
6	+	+	+	+	+	+				+	−	−
11	+	+	+				+	−	−	+	−	−
12	+	+	+				+	−	−	+	−	−
13	+	+	+	+	+	+	+	−	−	+	−	−

NOTE. In all, 10^6 cells were used for each sample. +, DNA fragment detected; −, DNA fragment not detected.
[a] Sensitivity, 1 copy/10^6 cells.
[b] Sensitivity, 10 copies/10^6 cells.
[c] Limit of detection, 30 pg/mL.

CD8 cells, can undergo a phenotypic conversion from $CD45RO^+$ $CD45RA^-$ to $CD45RO^{-/lo}$ $CD45RA^+$. These cells retain other phenotypic and functional features of memory cells [53–57], and this conversion thus represents a limited phenotypic change and not a reversion to a naive status. Thus, CD4 memory cells secrete the hallmark cytokines IL-4 and interferon (IFN)–γ. IFN-γ is also a hallmark of CD8 memory cells.

During chronic infections such as HIV, $CD45RO^{-/lo}$ $CD45RA^+$ cells may comprise the majority of CD8 memory cells [53–55] (L.J.P., unpublished data). Thus, although most $CD45RO^+$ CD4 memory cells will be eliminated with the anti-CD45RO IT, not all CD8 memory cells will be eliminated [51]. When HIV-infected patients undergo HAART, $CD45RO^{-/lo}$ naive cells increase in some but not all patients [58–61] (L.J.P., unpublished data). In addition, although we have good evidence that this IT spares $CD45RA^+$ cells [34], it will be important also to examine the effect of the IT on the function of $CD45RA^+$ naive CD4 and CD8 T cells.

With regard to the theoretical advantage of using ITs in HIV-positive patients, the elimination of accessible T cells in the lymph nodes, blood, and bone marrow (compared with previous clinical use of ITs to decrease inaccessible tumor nodules) should require smaller doses of the ITs than those required for cancer patients [50]. Although the toxicity of ITs in HIV-positive persons is unknown, in phase 1 trials with lymphoma patients [50], the most common adverse events have been manifestations of vascular leak syndrome (VLS). VLS was most severe and dose limiting in patients who had undergone prior radiotherapy, which would be unlikely in HIV-positive persons. At doses predicted to eliminate $CD45RO^+$ cells, we would be well below this dose. Furthermore, we now have generated a recombinant–ricin A chain (rRTA) in which the sequences responsible for VLS have been altered (E.S.V., unpublished data). ITs prepared with this rRTA are being evaluated. Of importance, latent HIV disease is similar to minimal residual neoplastic disease in representing an ideal setting for IT therapy. Nevertheless, unpredicted toxicities might occur and these can only be studied in phase 1 clinical trials.

Acknowledgments

We thank R. Doug Hardy and the patients and staff of the Amelia Court Clinic at Parkland Hospital, Dallas, for collaboration; Jeanine Hatfield, Christine Pitcher, and Joshua Walker, for technical assistance and advice; S. Flowers, for secretarial assistance; and J. Uhr, V. Garcia, and D. Margolis, for helpful comments on the manuscript.

References

1. Gulick RM, Mellors JW, Havlir D, et al. Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N Engl J Med **1997**;337:734–9.
2. Chun TW, Carruth L, Finzi D, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature **1997**;387:183–8.
3. Finzi D, Blankson J, Siliciano JD, et al. Latent infection of $CD4^+$ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med **1999**;5:512–7.
4. Finzi D, Hermankova M, Pierson T, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **1997**;278:1295–300.
5. Chun TW, Stuyver L, Mizell SB, et al. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci USA **1997**;94:13193–7.
6. Wong JK, Hezareh M, Gunthard HF, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **1997**;278:1291–5.
7. Zhang L, Ramratnam B, Tenner-Racz K, et al. Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. N Engl J Med **1999**;340:1605–13.
8. García F, Plana M, Vidal C, et al. Dynamics of viral load rebound and immunological changes after stopping effective antiretroviral therapy. AIDS **1999**;13:F79–86.
9. Siliciano RF. Latency and reservoirs for HIV-1. AIDS **1999**;13:S49–58.
10. Quayle AJ, Xu C, Mayer KH, Anderson DJ. T lymphocytes and macrophages, but not motile spermatozoa, are a significant source of human immunodeficiency virus in semen. J Infect Dis **1997**;176:960–8.
11. Zhang H, Dornadula G, Beumont M, et al. Human immunodeficiency virus type 1 in the semen of men receiving highly active antiretroviral therapy. N Engl J Med **1998**;339:1803–9.
12. Brooks DG, Kitchen SG, Kitchen CMR, Scripture-Adams DD, Zack JA. Generation of HIV latency during thymopoiesis. Nat Med **2001**;7:459–64.

13. Chun TW, Finzi D, Margolick J, Chadwick K, Schwart D, Siliciano RF. In vivo fate of HIV-1–infected T cells: quantitative analysis of the transition to stable latency. Nat Med **1995**; 1:1284–90.
14. Bukrinsky MI, Stanwick TL, Dempsey MP, Stevenson M. Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection. Science **1991**; 254:423–7.
15. Blankson JN, Finzi D, Pierson TC, et al. Biphasic decay of latently infected $CD4^+$ T cells in acute human immunodeficiency virus type 1 infection. J Infect Dis **2000**; 182:1636–42.
16. Grossman Z, Polis M, Feinberg MB, et al. Ongoing HIV dissemination during HAART. Nat Med **1999**; 5:1099–104.
17. Ramratnam B, Mittler JE, Zhang L, et al. The decay of the latent reservoir of replication-competent HIV-1 is inversely correlated with the extent of residual viral replication during prolonged anti-retroviral therapy. Nat Med **2000**; 6:82–5.
Perelson AS, Essunger P, Cao Y, et al. Decay characteristics of HIV-1–infected compartments during combination therapy. Nature **1997**; 387: 188–91.
19. Blankson J, Siliciano RF. Interleukin 2 treatment for HIV infection. JAMA **2000**; 284:236–8.
20. Chun TW, Engel D, Mizell SB, et al. Effect of interleukin-2 on the pool of latently infected, resting of $CD4^+$ T cells in HIV-1 infected patients receiving highly active anti-retroviral therapy. Nat Med **1999**; 5:651–5.
21. Hengge UR, Goos M, Esser S, et al. Randomized controlled phase II trial of subcutaneous interleukin-2 in combination with highly active antiretroviral therapy (HAART) in HIV patients. AIDS **1998**; 12:F225–34.
22. Davey RT, Murphy RL, Graziano SL, et al. Immunologic and virologic effects of subcutaneous interleukin 2 in combination with antiretroviral therapy: a randomized controlled trial. JAMA **2000**; 284:183–9.
23. Saag MS, Kilby JM. HIV-1 and HAART: a time to cure, a time to kill. Nat Med **1999**; 5:609–11.
24. Borvak J, Chou CS, Van Dyke G, et al. The use of cyclosporine, FK506, and SDZ NIM811 to prevent $CD25^-$ quiescent peripheral blood mononuclear cells from producing human immunodeficiency virus. J Infect Dis **1996**; 174:850–3.
25. Ravot E, Lisziewicz J, Lori F. New uses for old drugs in HIV infection: the role of hydroxyurea, cyclosporin and thalidomide. Drugs **1999**; 58: 953–63.
26. Rizzard GP, Capilupp B, Chave JP, et al. Activity of cyclosporin A in combination with highly active antiretroviral therapy in primary HIV-1 infection. AIDS **2000**; 14(Suppl 4):S12.
27. Bell KD, Ramilo O, Vitetta ES. Combined use of an immunotoxin and cyclosporine to prevent both activated and quiescent peripheral blood T cells from producing type 1 human immunodeficiency virus. Proc Natl Acad Sci USA **1993**; 90:1411–5.
28. Barlett JA, Silberman M, Miralles GD, et al. Antiretroviral therapy plus cyclophosphamide to diminish HIV DNA in lymphoid tissue [abstract 16]. In: Program and abstracts of the 8th Conference on Retrovirus and Opportunistic Infections (Chicago). Alexandria, VA: Foundation for Retrovirology and Human Health, **2001**.
29. Hel Z, Venzon D, Poudyal M, et al. Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. Nat Med **2000**; 6:1140–6.
30. Weissman D, Ni H, Scales D, et al. HIV *gag* mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response. J Immunol **2000**; 165:4710–7.
31. Barouch D, Santra S, Schmitz JE, et al. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science **2000**; 290:486–92.
32. Ramilo O, Bell KD, Uhr JW, Vitetta ES. Role of $CD25^+$ and $CD25^-$ T cells in acute HIV infection in vitro. J Immunol **1993**; 150:5202–8.
33. Borvak J, Chou C, Bell KD, et al. Expression of CD25 defines peripheral blood mononuclear cells with productive versus latent HIV infection. J Immunol **1995**; 155:3196–204.
34. McCoig C, Van Dyke G, Chou CS, Picker LJ, Ramilo O, Vitetta ES. An anti-CD45RO immunotoxin eliminates T cells latently infected with HIV-1 in vitro. Proc Natl Acad Sci USA **1999**; 96:11482–5.
35. Chou CS, Ramilo O, Vitetta ES. Highly purified $CD25^-$ resting T cells cannot be infected de novo with HIV-1. Proc Natl Acad Sci USA **1997**; 94:1361–5.
36. Bonnefoix T, Bonnefoix P, Verdiel P, Sotto JJ. Fitting limiting dilution experiments with generalized linear models in a test of the single-hit Poisson assumption. J Immunol Methods **1996**; 194:113–9.
37. Layton JE, Vitetta ES, Uhr JW, Krammer PH. Clonal analysis of B cells induced to secrete IgG by T cell–derived lymphokine(s). J Exp Med **1984**; 160:1850–63.
38. Macken C. Design and analysis of serial limiting dilution assays with small sample sizes. J Immunol Methods **1999**; 222:13–9.
39. Ghetie V, Thorpe P, Ghetie MA, Knowles P, Uhr JW, Vitetta ES. The GLP large scale preparation of immunotoxins containing deglycosylated ricin A chain and a hindered disulfide bond. J Immunol Methods **1991**; 142:223–30.
40. Smith SH, Brown MH, Rowe D, Callard RE, Beverley PC. Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. Immunology **1986**; 58:63–70.
41. Engert A, Martin G, Amlot P, Wijdenes J, Diehl V, Thorpe P. Immunotoxins constructed with anti-CD25 monoclonal antibodies and deglycosylated ricin A–chain have potent anti-tumour effects against human Hodgkin cells in vitro and solid Hodgkin tumors in mice. Int J Cancer **1991**; 49: 450–6.
42. Pierson T, Hoffman TL, Blankson J, et al. Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. J Virol **2000**; 74:7824–33.
43. Collins RH Jr, Sackler CJ, Pitcher SL, et al. Immune reconstitution with donor-derived memory/effector T cells after orthotopic liver transplantation. Exp Hematol **1997**; 25:147–59.
44. Folks TM, Powell D, Lightfoote M, et al. Biological and biochemical characterization of a cloned Leu-3–cell surviving infection with the acquired immune deficiency syndrome retrovirus. J Exp Med **1986**; 164:280–90.
45. Bucy RP, Hockett RD, Derdeyn CA, et al. Initial increase in blood $CD4^+$ lymphocytes after HIV antiretroviral therapy reflects redistribution from lymphoid tissues. J Clin Invest **1999**; 103:1391–8.
46. Cohen OJ, Pantaleo G, Schwartzentruber DJ, Graziosi C, Vaccarezza M, Fauci AS. Pathogenic insights from studies of lymphoid tissue from HIV-infected individuals. J Acquir Immune Defic Syndr Hum Retrovirol **1995**; 10(Suppl 1):6–14.
47. Spina CA, Prince HE, Richman DD. Preferential replication of HIV-1 in the CD45RO memory cell subset of primary CD4 lymphocytes in vitro. J Clin Invest **1997**; 99:1774–85.
48. Davey RT Jr, Bhat N, Yoder C, et al. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. Proc Natl Acad Sci USA **1999**; 96:15109–14.
49. Rosenberg ES, Altfeld M, Poon SH, et al. Immune control of HIV-1 after early treatment of acute infection. Nature **2000**; 407:523–6.
50. Sausville EA, Vitetta ES. Clinical studies with deglycosylated ricin A–chain immunotoxins. In: Grossbard ML, ed. Monoclonal antibody–based therapy of cancer. Boston: Marcel Dekker, **1997**:81–9.
51. Wills MR, Carmichael AJ, Weekes MP, et al. Human virus–specific $CD8^+$ CTL clones revert from $CD45RO^{high}$ to $CD45RA^{high}$ in vivo: $CD45RA^{high}$ $CD8^+$ T cells comprise both naive and memory cells. J Immunol **1999**; 162:7080–7.
52. Komanduri KV, Viswanathan MN, Wieder ED, et al. Restoration of cytomegalovirus-specific $CD4^+$ T-lymphocyte responses after ganciclovir

and highly active antiretroviral therapy in individuals infected with HIV-1. Nat Med 1998;4:953–6.

53. Kern F, Khatamzas E, Surel I, et al. Distribution of human CMV–specific memory T cells among the CD8$^+$ subsets defined by CD57, CD27, and CD45 isoforms. Eur J Immunol 1999;29:2908–15.
54. Hamann D, Baars PA, Rep MH, et al. Phenotypic and functional separation of memory and effector human CD8$^+$ T cells. J Exp Med 1997; 186:1407–18.
55. Roederer M, Dubs JG, Anderson MT, Raju PA, Herzenberg LA. CD8 naive T cell counts decrease progressively in HIV-infected adults. J Clin Invest 1995;95:2061–6.
56. Rabin RL, Roederer M, Maldonado Y, Petru A, Herzenberg LA. Altered representation of naive and memory CD8 T cell subsets in HIV-infected children. J Clin Invest 1995;95:2054–60.
57. Okumura M, Fujii Y, Inada K, Nakahara K, Matsuda H. Both CD45RA$^+$ and CD45RA$^-$ subpopulations of CD8$^+$ T cells contain cells with high levels of lymphocyte function-associated antigen–1 expression, a phenotype of primed T cells. J Immunol 1993;150: 429–37.
58. Autran B, Carcelain G, Li TS, et al. Positive effects of combined antiretroviral therapy on CD4$^+$ T cell homeostasis and function in advanced HIV disease. Science 1997;277:112–6.
59. Pakker NG, Notermans DW, DeBoer RJ, et al. Biphasic kinetics of peripheral blood T cells after triple combination therapy in HIV-1 infection: a composite of redistribution and proliferation. Nat Med 1998;4: 208–14.
60. Zhang ZQ, Notermans DW, Sedgewick G, et al. Kinetics of CD4$^+$ T cell repopulation of lymphoid tissues after treatment of HIV-1 infection. Proc Natl Acad Sci USA 1998;95:1154–9.
61. Pakker NG, Kroon ED, Roos MT, et al. Immune restoration does not invariably occur following long-term HIV-1 suppression during antiretroviral therapy. INCAS Study Group. AIDS 1999;13:203–12.

RECEIVED
APR 15 2002
TECH CENTER 1600/2900

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Daniel A. Vallera et al. Art Unit : 1643
Serial No. : 09/579,738 Examiner : E. Sorbello
Filed : May 26, 2000
Title : CELL-MEDIATED TARGETING OF TOXINS TO PATHOGENIC CELLS

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF DANIEL A. VALLERA UNDER 37 C.F.R. §1.132

I, DANIEL A. VALLERA, declare:

1. I am an inventor of the subject matter in the above-mentioned patent application.

2. I have read and understood the Office Action dated September 14, 2001, in which claims 1-43 were rejected as being unpatentable for lack of enablement.

3. The attached figures in Appendix A show the results of *in vivo* experiments carried out under my supervision.

The details of the experiments are as follows.

Genetic constructs encoding two immunotoxic fusion proteins containing, as targeting domains, the cytokine interleukin-3 (IL-3) and as toxic domains either DT390 (amino acids 1-390 of diphtheria toxin) or the BAX proaptotic protein were generated and separately subcloned into the LNCX retroviral expression vector in which the *neo* gene is replaced with a nerve growth factor receptor-encoding nucleic acid sequence, thereby creating the recombinant retroviral expression vectors LNCX.IL3-DT and LNCX.IL-3-BAX, respectively. These two fusion protein-encoding vectors were separately transduced into the murine CD4+ T lymphocyte line C8, which is specific for the IL-3 receptor-expressing murine myeloid leukemia cell line

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D. C. 20231, on the date set forth below.

Signed Maldonado
Name GINA MALDONADO
Date 3/27/02

FBL3, produces multiple cytokines, and is cytotoxic. Succesfully transduced C8 cells were identfied by their cell-surface expression of NGFR which was detected by fluorescence flow cytometry using an NGFR-specific antibody conjugated with fluorescein isothiocyanate (FITC). The resulting recombinant cell lines were cloned by limiting dilution and a clone of each, designated C8.IL3-DT and C8.IL3-BAX, was selected for further experimentation. All the above procedures were carried out essentially as described in Example 1 of the present patent application.

Figures 1 and 2 in Appendix A are survival curves obtained from two separate experiments in which mice (5 to 7 per group) were injected intraperitoneally (i.p.) with about 5 x 10^6 FBL3 cells. Four days later the mice were injected i.p. with about 5 x 10^6 C8.IL3-DT cells or control untransduced C8 cells. As can be seen from Figures 1 and 2, the C8.IL3-DT cells ("IL3DT retIT") caused a significant increase in survival time compared to control untransduced C8 cells ("Non-transduced"). Mice injected with FBL3 cells but no T cells (data not shown) died as fast as the mice injected with FBL3 cells and control untransduced C8 cells.

Figures 3 and 4 in Appendix A are survival curves obtained from two separate experiments in which mice (5-7 per group) were injected i.p. with about 5 x 10^6 FBL3 cells. Four days later the mice were injected i.p. with about 5 x 10^6 C8.IL3-BAX cells or control untransduced C8 cells. As can be seen from Figures 3 and 4, the C8.IL3-BAX cells ("IL3BAX retIT") caused a significant increase in survival time compared to control untransduced C8 cells ("Non-transduced"). Mice injected with FBL3 cells but no T cells (data not shown) died as fast as the mice injected with FBL3 cells and control untransduced C8 cells.

The above data show that CTL secreting immunotoxic fusion proteins containing as a targeting domain the cytokine IL-3 and as toxic domains DT390 or BAX have *in vivo* therapeutic effects on a systemic leukemia.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 3/13/02

Daniel A. Vallera

Fig. 1

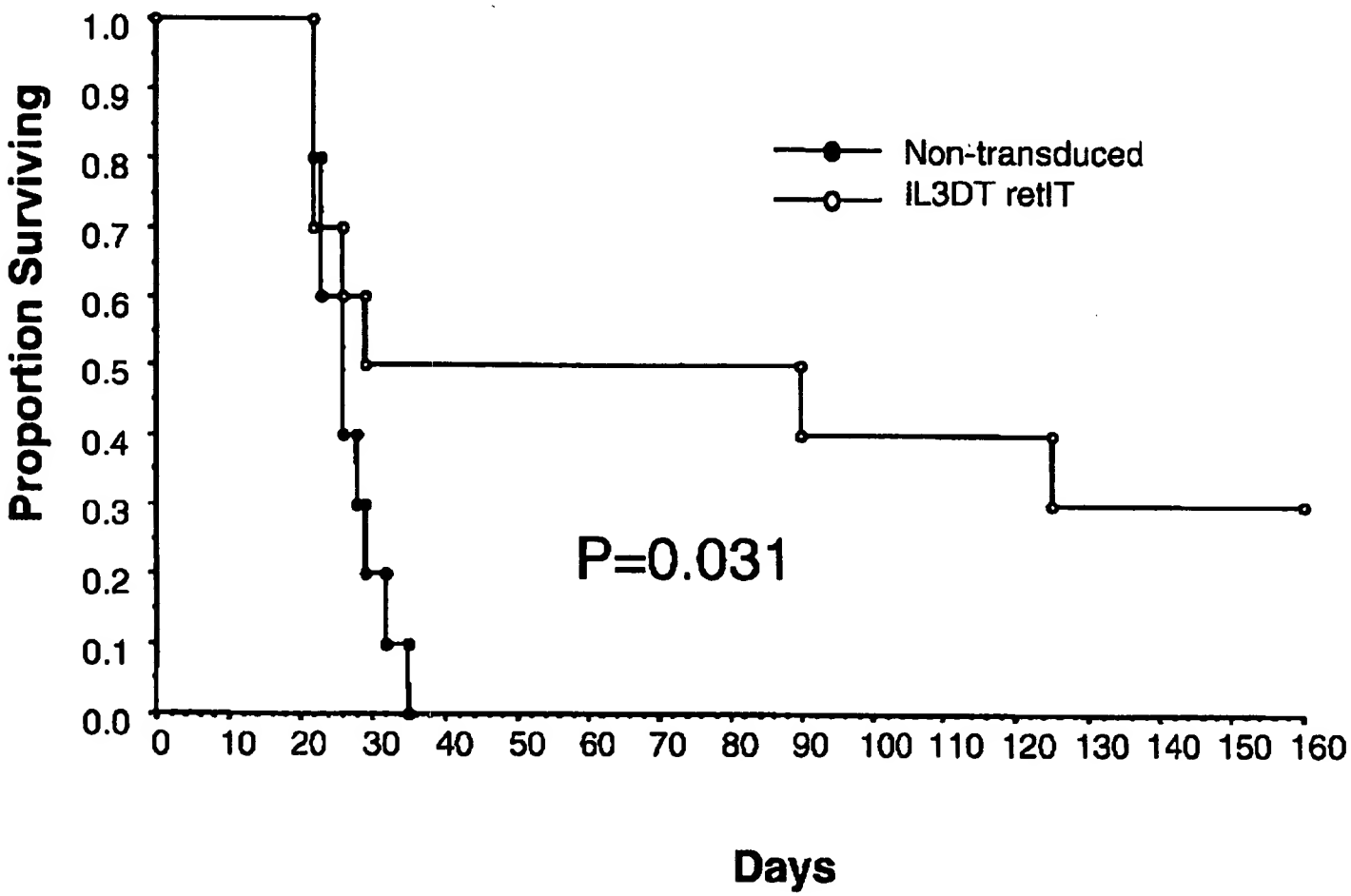

Proportion Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
Days
Non-transduced
IL3DT retIT
P=0.031

Fig. 2

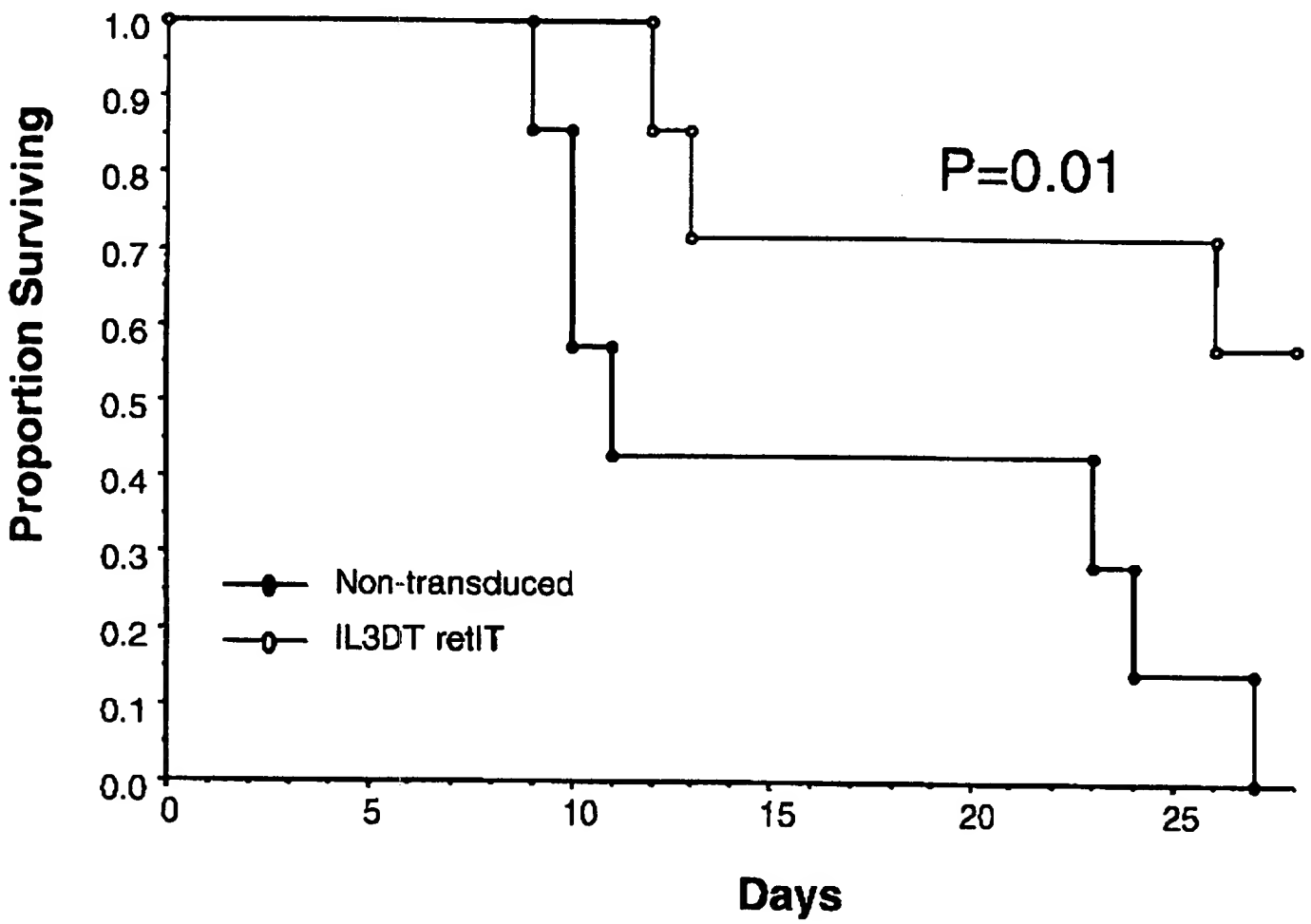

Proportion Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
Days
P=0.01
Non-transduced
IL3DT retIT

Fig. 3

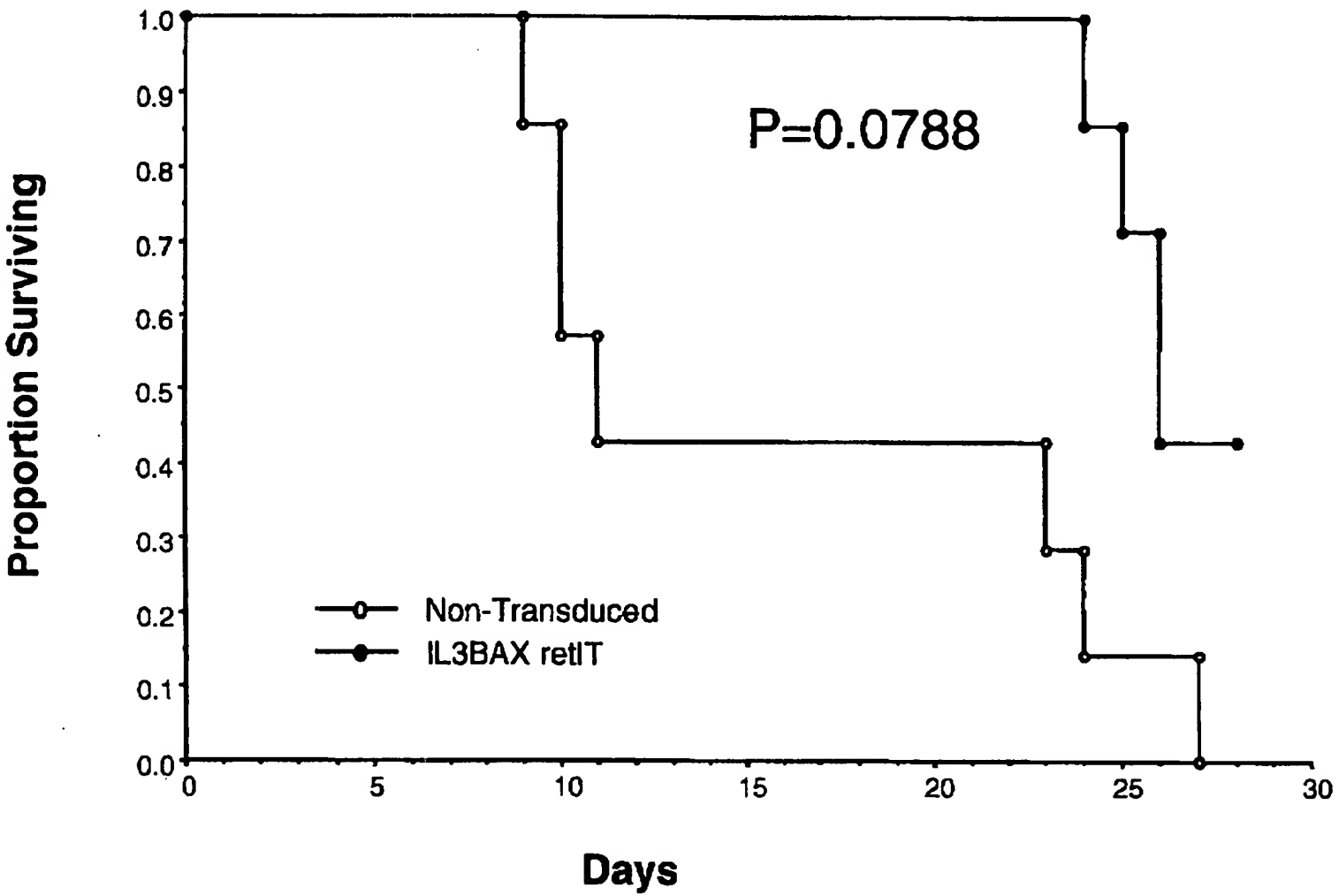
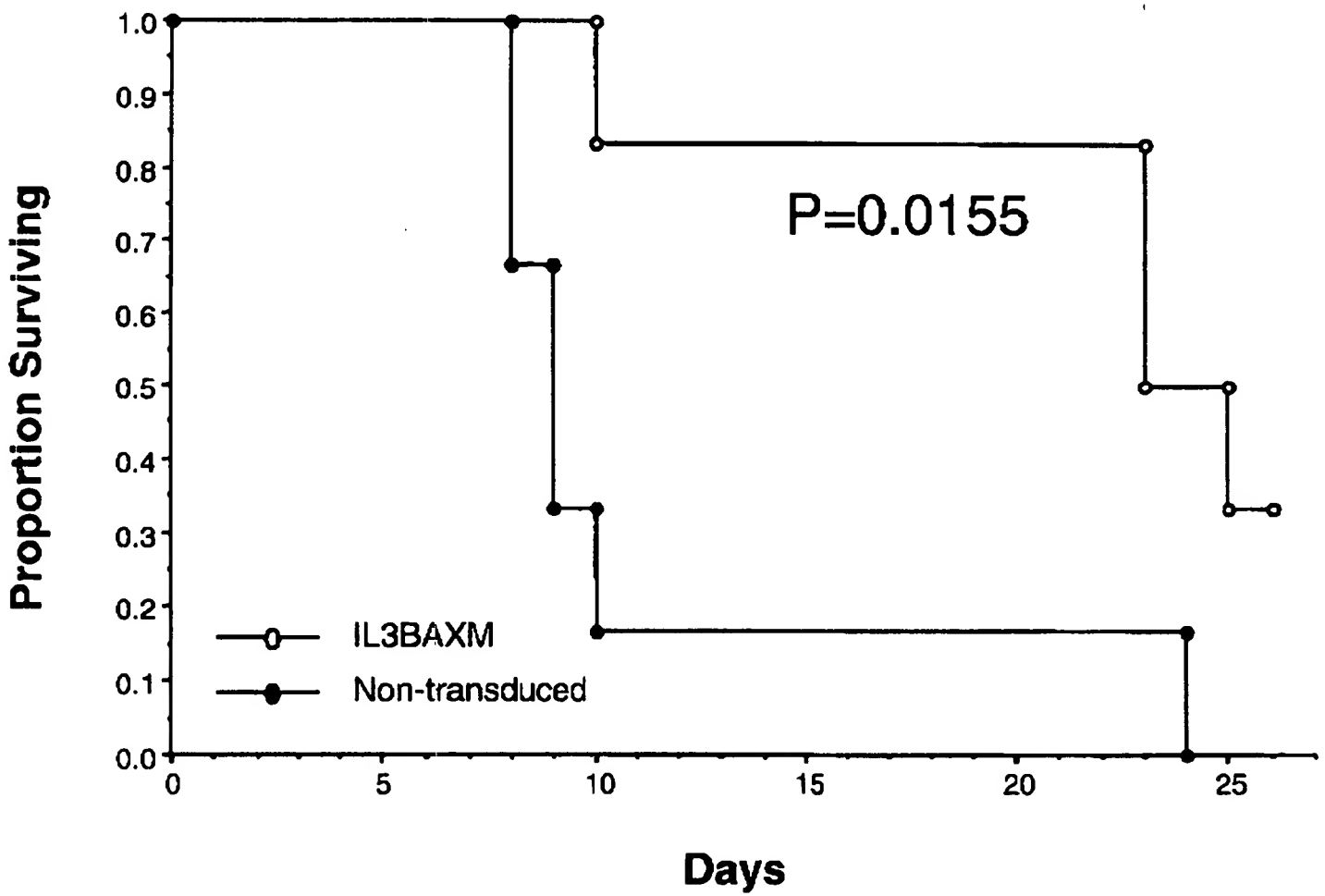

Proportion Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
P=0.0788
Non-Transduced
IL3BAX retIT
0
5
10
15
20
25
30
Days

Fig. 4


Proportion Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
P=0.0155
IL3BAXM
Non-transduced
0
5
10
15
20
25
Days